ABSTRACTS:

Poster Presentations

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Microtubules: Centrosomes and Centrioles

P1000/B1

PLK4 employs its cryptic polo box to scaffold the formation of pseudocentrosomes that accelerate acentriolar spindle assembly.

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Segregation of replicated chromosomes during mitosis requires rapid assembly of a bipolar microtubule-based spindle. In metazoans, this is accomplished by a pair of centrosomes, composed of a centriolar core surrounded by a pericentriolar matrix, that catalyzes the assembly of spindle microtubules. In human cells that lack centrioles due to chemical inhibition of the centriole duplication kinase PLK4, spindle assembly occurs through slow self-organization of pericentriolar matrix containing foci, which takes about twice as long as in cells with centrosomes. Loss of TRIM37, a centrosome-regulating ubiquitin ligase, restores rapid bipolar spindle assembly in centrinone-treated acentriolar cells by enabling the formation of an array of foci that function as pseudocentrosomes. Pseudocentrosomes nucleate microtubules and robustly cluster to form two spindle poles. Pseudocentrosomes contain proteins proximal to PLK4 in the centrosome including CEP192, CEP152, CEP63 and the centriolar satellite protein PCM1, and are reminiscent of PLK4-containing foci required for naturally occuring acentriolar spindle assembly during the early mitotic divisions of mouse embryos. Using inducible gene knockouts in centrinone-treated *TRIM37Δ* cells, we found that PLK4 is the only centrosomal component of eight tested that was required for pseudocentrosome formation. The centrosomal scaffold protein CEP192, while dispensable for pseudocentrosome formation, was essential for their function; by contrast, the major pericentriolar matrix scaffold protein CDK5RAP2 was dispensable for both pseudocentrosome formation and function. To determine how PLK4 supports pseudocentrosome formation, we replaced endogenous PLK4 with mutants engineered to disrupt four of its known features: (a) kinase activity; (b) basic crater in the cryptic-polo box (CPB) domain that interfaces with centriolar receptors; (c) CPB homodimerization; and (d) oligomerization mediated by a C-terminal Polo Box domain. This analysis revealed that point mutations in the CPB basic crater prevented pseudocentrosome formation; by contrast, single or double depletion of the centriolar receptors CEP192 and CEP152 that bind to the basic crater did not. Thus, the PLK4 CPB basic crater is essential to form assemblies that recruit CEP192 and other components to build pseudocentrosomes that generate spindle microtubules. We speculate that PLK4-scaffolded pseudocentrosomes are analogous to structures that promote acentriolar spindle assembly in specific natural contexts, such as oocyte meiosis, and may serve as a proxy for investigating the pericentriolar matrix-independent pathway for spindle microtubule generation that supports robust spindle assembly in human cells.

P1001/B2

Deciphering the Cell Cycle's Role in Regulating Centrosome Dynamics - Lessons From Mathematical Modelling.

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Centrosomes form when centrioles recruit an amorphous matrix of pericentriolar material (PCM) around themselves. Studies in flies and worms have revealed that Spd-2/SPD-2, Polo/PLK-1 and Cnn/SPD-5 (fly/worm nomenclature) cooperate to assemble a macromolecular scaffold that expands around the centrioles during mitosis; most, if not all, other mitotic PCM proteins ultimately assemble upon this scaffold. In both systems, Spd-2/SPD-2 appears to recruit Polo/PLK-1, which then phosphorylates Cnn/SPD-5 at multiple sites to stimulate the assembly of a Cnn/SPD-5 scaffold. In flies, centrioles appear to generate a local pulse of Polo activity, which is required to activate Spd-2's ability to recruit Polo, and so initiate mitotic PCM assembly. Here, we derive a spatio-temporal mathematical model to describe how the core embryonic Cdk/Cyclin-dependent cell cycle oscillator coordinates mitotic PCM assembly and disassembly in the early Drosophila embryo. We demonstrate that oscillations in Cdk/Cyclin activity translate into a switch-like behaviour that initiates Cnn-scaffold assembly at moderate levels of activity, maintains the Cnn-scaffold at high levels of activity, and then drives the rapid disassembly of the Cnn-scaffold when Cdk/Cyclin activity drops precipitously at the end of mitosis. We use several experimental approaches to derive estimates for all the key reaction parameters in our mathematical model, and we show that this model captures well the behaviour of the key centrosome scaffolding proteins. By exploring the parameter space of the viscoelastic model, we can predict how the mechanical behaviour of the system varies between a liquid-like and solid-like regime. These studies have the potential to shed important light on the biophysical nature of mitotic centrosomes—in particular whether liquid-liquid phase separation is an important driver of mitotic PCM assembly—a topic that is hotly debated.

P1002/B3

A disease-associated PPP2R3C-MAP3K1 phospho-regulatory module controls centrosome function.

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Centrosomes have critical roles in microtubule organization and cell signaling that underlie their importance in development and disease. However, the mechanisms that regulate centrosome function are not fully defined, and thus how defects in centrosomal regulation contribute to disease is incompletely understood. Using a systems genetics approach, we find here that PPP2R3C, a PP2A phosphatase subunit, is a novel distal centriole protein and functional partner of centriolar proteins CEP350 and FOP. We further show that a key function of PPP2R3C and FOP is to counteract the kinase activity of MAP3K1. In support of this model, *MAP3K1* knockout suppresses growth defects caused by inactivation of *PPP2R3C* or *FOP*, and MAP3K1 and PPP2R3C have opposing effects on basal and microtubule stress-induced JNK signaling. Illustrating the importance of balanced MAP3K1 and PPP2R3C activities, acute overexpression of MAP3K1 severely inhibits centrosome function and triggers rapid disintegration of centrioles. Highlighting the physiologic significance of these findings, inactivating *PPP2R3C* mutations and activating *MAP3K1* mutations both cause congenital syndromes characterized by gonadal dysgenesis. As a syndromic *PPP2R3C* variant is defective in centriolar localization and binding to centriolar protein FOP, we propose that imbalanced activity of this centrosomal kinase-phosphatase pair is the shared cause of these disorders. Lastly, we note that a functional ortholog of the PP2A-PPP2R3C-FOP-CEP350 complex regulates the microtubule cytoskeleton in plants, which lack centrioles entirely. Thus, our findings reveal an evolutionary ancient module that has been conserved across highly divergent modes of microtubule organization. Collectively, our study identifies a new centrosomal phospho-regulatory module, sheds light on disorders of gonadal development, and illustrates the power of systems genetics to reveal previously unrecognized gene functions.

P1003/B4

Microtubule-Based Condensate Transport Contributes To Centrosome Clustering In Cancer Cells.

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The centrosome, a membrane-less organelle, undergoes amplification in various cancers, contributing to aneuploidy. Cancer cells prevent multipolar spindle formation and subsequent apoptotic cell death by clustering extra centrosomes into a pseudo-bipolar spindle, ensuring accurate cell division. Our study demonstrates that at mitotic entry, HSET, a low-processivity kinesin motor, facilitates transport and coalescence of centrosome-like condensates formed by CDK5RAP2, thereby reducing cytoplasmic puncta. Subsequently, these condensates allocate to form the spindle poles. Total internal reflection fluorescence (TIRF)-based in vitro reconstitution experiments reveal that HSET and CDK5RAP2 co-condensate, enhancing their processivity on microtubules. HSET directly transports CDK5RAP2 condensates towards the microtubules minus ends, converging at interphase microtubule radical arrays and mitotic spindle poles in animal cells. Computer simulations suggest that elevated HSET number and processivity promote centrosome clustering in HSET-over-expressing cancers. Our findings highlight a microtubule-dependent mechanism that regulates the cellular localization of biomolecular condensates.

P1004/B5

Microtubule and centrosome remodeling in quiescence.

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Cells are constantly facing decision to proliferate or enter in a non-proliferating state. Quiescence is defined as a temporary absence of proliferation, and is the most widespread cellular state on Earth, ranging from prokaryotic to eukaryotic organisms. Quiescence establishment, maintenance and exit, by balancing cell proliferation, are key steps involved not only in normal development and tissues homeostasis, but also in major human pathologies such as cancers. Quiescence is at the heart of the aging process, as over time cells must overcome the deleterious effects of constant increase in damaged macromolecules while maintaining their ability to reproliferate. Recently, it has been shown that upon quiescence establishment, microtubules are reorganized and form a stable structure called Q-nMT bundle that is required for cell survival in quiescence and that controls the return into the proliferative state. We have investigated the relationship between the Q-nMT bundle and the centrosome from which it originates. I have shown that the yeast centrosome is modified upon quiescence establishment, with an increased recruitment of gamma- TuRC components. We are currently searching for possible post-translational modifications of gamma-TuRC and partners that may be involved in Q-nMT bundle formation. Finally, upon exit from quiescence, I have shown that the Q-nMT bundle disassembly is mandatory for centrosome separation upon re-entry into mitosis. We propose that, like primary cilia in metazoans, the Q-nMT bundle may act as a checkpoint to allow quiescence exit, thereby opening new avenues for understanding the interdependence between the primary cilium and quiescence.

P1005/B6

NuSAP Mediates CEP57 Recruitment for Proper Centriole Engagement.

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The precise coordination of centrosome function and mitotic spindle formation is essential for accurate cell division and chromosome segregation in vertebrates. While the role of microtubule-associated proteins (MAPs) in spindle formation is well established, their contribution to centrosome integrity remains underexplored. Nucleolar and Spindle-Associated Protein (NuSAP), a RanGTP-regulated MAP, is known for its critical function in spindle stability and chromosome segregation, but its role in maintaining centrosomal integrity has not been fully characterized. In this study, we demonstrate that NuSAP depletion leads to multipolar spindle formation and premature centriole disengagement, uncovering a novel function of NuSAP at the centrosome. Our findings reveal that NuSAP localizes to centrosomes and interacts with CEP57, a key pericentriolar material (PCM) scaffolding protein. Depletion of NuSAP destabilizes centriole tubulin structure, resulting in mis-localization of CEP57, PCM disorganization, and subsequent centriole disengagement. This study highlights the essential role of NuSAP in anchoring the CEP57 complex to centrioles and underscores the previously unrecognized involvement of MAPs in maintaining centrosome integrity, providing new insights into the molecular mechanisms governing cell division.

P1006/B7

Structural Organization of the Centriole Lumen by the Inner Scaffold Network.

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Centrosomes are the primary microtubule-organizing centers (MTOCs) in eukaryotes, playing crucial roles in cell proliferation, polarity, and ciliogenesis. Centrosomes consist of the centriole, a nine-fold symmetrical microtubule-based cylinder, and the pericentriolar material (PCM). The function of centrosomes is closely tied to centriole integrity. Therefore, defects in centriolar structures can lead to various centrosome-linked diseases, including cancer, neurodevelopmental disorders, and ciliopathies. The inner scaffold is a ring-like substructure in the centriolar lumen that contributes to centriolar integrity. Based on their localization, centriolar proteins such as POC1B, POC5, FAM161A, and CCDC15 have been identified as potential components of the inner scaffold. In this study, we demonstrate that the two human paralogues, POC1A and POC1B, are essential for the proper localization and function of other inner scaffold proteins. Both POC1 proteins share a similar domain architecture, consisting of an N-terminal WD40 domain and a C-terminal coiled coil. These domains facilitate interactions with specific subsets of proteins. The core of the inner scaffold is formed by a WD40-mediated interaction between POC1A and the POC5-centrin complex, which is an elongated structure consisting of four POC5 molecules. Additionally, POC1A and POC1B form a heterodimer through their coiled coils, cross-linking the inner scaffold and anchoring it to the microtubule wall. Loss of either POC1A or POC1B results in mislocalization of inner scaffold proteins and affects centriolar stability, leading to short and fragmented centrioles, similar to the phenotype observed in *POC5*$^{-/-}$ cells. This ultimately leads to mitotic defects. Our data indicate how the WD40 domain and coiled coil region in the POC1 proteins mediate different

interaction patterns and how the inner scaffold is involved in maintaining centriolar stability, emphasizing the importance of centriole structural integrity for centrosome function.

P1007/B8

Architecture of the centriolar A-C linker.

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Centrioles are massive protein assemblies at the cores of centrosomes and basal bodies. A hallmark of centriolar architecture is the A-C linker, which connects the A- and C-tubules of neighbouring microtubule triplets. Despite its identification several decades ago, we do not understand how the A-C linker is built, nor how it crosslinks microtubules in the centriole. Here, we report high-resolution cryo-electron microscopy reconstructions of the native A-C linker bound to centriolar microtubule triplets in *T. thermophila* basal bodies. We discovered that the A-C linker adopts two distinct conformations: an extended form in the proximal centriolar microtubule triplet region, and a narrow form in the distal portion. These conformations are distinguished by unique A- and C-tubule-binding proteins and an alpha-helical bundle that protrudes from the proximal A-C linker into the cell body. We identified several core A-C linker components, which together repeat every 8 nm along the centriole axis via a fishbone-like alpha-helical lattice. The structure of adjacent A and C-tubules shows that the A-C linker forms a bridge with novel triplet-specific B-C junction proteins, allowing us to build the first molecular model of a centriolar microtubule triplet pair. Our results uncover surprising structural and biochemical heterogeneity in centriole-associated complexes and suggest how the A-C linker could confer mechanical properties to centrioles in motile cells.

P1008/B9

A delta-tubulin/epsilon-tubulin/Ted protein complex is required for centriole architecture.

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Centrioles have a unique, conserved architecture formed by three linked “triplet” microtubules arranged in nine-fold symmetry. The mechanisms by which these triplet microtubules are formed are not understood, but likely involve the noncanonical tubulins delta-tubulin and epsilon-tubulin. Previously, we found that human cells deficient in delta-tubulin or epsilon-tubulin form abnormal centrioles, characterized by an absence of triplet microtubules, lack of central core protein POC5, and a futile cycle of centriole formation and disintegration. Here, we show that human cells lacking either of the associated proteins TEDC1 and TEDC2 have these same phenotypes. Using ultrastructure expansion microscopy, we identified the roles of these proteins and triplet microtubules in centriole architecture by mapping the locations of centriolar proteins throughout the cell cycle. We find that mutant centrioles have normal architecture during S-phase. By G2-phase, mutant centrioles grow to the same length as control centrioles, but fail to recruit inner scaffold proteins of the central core. Instead, the inner lumen of centrioles is filled with an expanded proximal region, indicating that these proteins, or the triplet microtubules themselves, may be required for recruiting central core proteins and restricting the length of the proximal end. During mitosis, the mutant centrioles elongate further before fragmenting and disintegrating. All four proteins physically interact and TEDC1 and TEDC2 are capable of interacting in the absence of the tubulins. These results support an AlphaFold Multimer structural prediction model

for the tetrameric complex, in which delta-tubulin and epsilon-tubulin are predicted to form a heterodimer. TEDC1 and TEDC2 localize to centrosomes and are mutually dependent on each other and on delta-tubulin and epsilon-tubulin for localization. These results indicate that delta-tubulin, epsilon-tubulin, TEDC1, and TEDC2 function together in promoting robust centriole architecture. This work also lays the groundwork for future dissection of this complex, which will provide a basis for determining the mechanisms that underlie the assembly and interplay between compound microtubules and inner centriole structure.

P1009/B10

Dissecting the dynamics of human centriole assembly by correlative cell cycle-centriole cycle ultrastructural analysis.

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The canonical human centriole is built of nine sets ofmicrotubule triplets, the assembly of which, in proliferating cells, starts inthe early S phase immediately after the initiation of nascent centrioles(procentrioles). Procentriole's longitudinal growth continues throughout itsfirst interphase and completes in its second cell cycle. The availableultrastructural analyses indicate that microtubule triplets are present alreadyin procentriole's first mitosis. The correlation of various centriolarcomponents with centriole length, irrespective of the exact cell cycle stages,has recently been published. However, the precise and systematic dissection ofthe dynamics of procentriole growth incorrelation with the cell cycle stages is still lacking. This gap infundamental knowledge needs to be filled to understand the mechanisms thatsynchronize the centriole assembly with the cell cycle. Such synchronizationoperates throughout the lifetime, ensuring centriole, centrosome, and ciliahomeostasis. Here, we report a study in which we systematically analyzedthe status of procentriole microtubules in precisely defined S and G2 cellcycle stages in several human cell lines. We employed a precise cell cycleproliferation marker and conducted correlative live and electron microscopyanalyses in combination with various super-resolution analyses. We found thatboth longitudinal and lateral incorporation of procentriole microtubules istied to cell cycle transitions and driven by the concerted action of severalkinases. We show that experimentally imbalanced activity of these kinases,which de-couples procentriole growth from cell cycle progression, results inthe loss of centrosome homeostasis. Our meticulous approach unravels thefundamental principles underlying centriole timely assembly in healthyproliferating cells.

P1010/B11

Reconstituting organelle assembly on the surface of synthetic beads.

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Almost all human cells are born with a single pair of centrioles, comprising an older mother and a closely-engaged younger daughter. In cycling cells, the centriole pair duplicates precisely once every cell cycle: mother and daughters disengage, the daughter matures into a mother, and the two mothers then each give birth to an engaged daughter that grows from their side during S-phase. In mitosis, the two centriole pairs recruit an amorphous matrix of pericentriolar material (PCM) to form two centrosomes that organise the poles of the mitotic spindle. Centrioles probably comprise >100 different types of protein, and mitotic centrosomes probably >400 different types of protein, yet these structures can

rapidly and precisely assemble during each cell cycle—always forming at the right time and place, and always growing to the right size. How do cells achieve this remarkable feat of bioengineering? Recent studies have shown that centriole and centrosome assembly is driven by a small "core" set of conserved proteins. Here we show that centriole and centrosome assembly can be reconstituted on the surface of synthetic beads that have been coupled to an anti-GFP nanobody and injected into *Drosophila* embryos expressing Sas-4-GFP, a core centriole/centrosome-assembly protein (CPAP in humans). These Sas-4-coated beads recruit mitotic PCM and nucleate MTs in synchrony with the endogenous centrosomes in the embryo. Remarkably, they also generate what appear to be *bona fide* centrioles that disengage from the bead surface, organise PCM and MTs, and then duplicate in synchrony with the endogenous centrioles. Thus, simply concentrating Sas-4 on a bead surface appears sufficient to initiate a robust self-assembly process in the embryo that can rapidly and efficiently build centrioles and centrosomes—in coordination with the cell cycle, but independent of the normal centriole template. These studies provide important insights into how cells can build such complicated protein machines so quickly and so accurately.

P1011/B12

Regulation of PCM in C. elegans sperm - a MTOC halo around sperm DNA?.

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During cell division, the centrosome acts as a microtubule organizing center (MTOC), nucleating and localizing microtubules through its pericentriolar material (PCM). Although its ability to act as an MTOC is a conserved feature of the centrosome, the molecular regulation of centrosomal MTOC function can vary depending on cell type. In *C. elegans,* the PCM is composed of three essential proteins, PCMD-1, SPD-2/Cep-192, and SPD-5, the functional homologue of CDK5RAP2/Cnn. We previously found that although these proteins are all essential during the first cell division in *C. elegans,* only SPD-5 is essential for MTOC function across cell types in the developing organism. **To understand how MTOC activity is regulated at the centrosome, we concentrated our efforts on understanding SPD-5 regulation in dividing cells**. One context pertinent to the study of PCM regulation is male meiosis. In *C. elegans,* spermatogenesis concludes with the elimination of the spindle pole in a cellular compartment, the residual body (RB), from which the spermatozoon buds off. This residual body effectively acts as a trashcan for the spermatozoon. However, how this process is made possible and how it relates to the typical stages of PCM disassembly remains a mystery. Using live imaging of endogenously tagged proteins, we observed that during spindle pole disassembly, the PCM fragments into smaller PCM complexes, or "packets." The packets remain attached to microtubules and migrate to the residual body, refocusing into a pole. Interestingly, these packets are not further disassembled in the residual body and remain stable through time. Surprisingly, we observed that soon after the spindle pole is eliminated from the spermatozoon, tubulin and SPD-5 progressively surround the sperm DNA, forming a "halo" around the sperm DNA. Interestingly, SPD-2 and PCMD-1 were only observed at the sperm centrioles and in the residual body, but not in the halo. We analyzed different proteins linked to MTOC activity using both live imaging and expansion microscopy and observed that a subset consistently localized similarly, surrounding the DNA. Using a combination of expansion microscopy and cryotomography, we are currently assessing the organization of this halo. Based on preliminary genetic data disrupting these halo proteins, we hypothesize that these sperm-provided proteins might contribute to controlling the timely activation of the centrosome into a spindle pole in the zygote.

P1012/B13

The *C. elegans*WASH complex supports transport and microtubule function.

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In *C. elegans*, only two Nucleation Promoting Factors (NPSs) that regulate branched actin through Arp2/3 have been examined, WAVE and WASP. We analyzed the orthologs of the third *C. elegans* Apr2/3 NPF, the WASH complex. The WASH complex is similar to the WAVE complex, in that both complexes include 5 paralogous components. Surprisingly, only 4 of the 5 components had been identified in *C. elegans*, and the role of WASH had not been previously described. We used existing mutations, RNAi and CRISPR to determine the effects of loss of the WASH complex, focusing on two epithelial tissues: the adult intestine and the embryonic epidermis. We used two phenotypic assays to test the role of WASH components, including a candidate for the missing 5th WASH component, the FAM21 proposed ortholog, CO5G5.2. (1) Using assays for protein transport showed that loss of any WASH component, using mutations or RNAi, resulted in the same phenotype: defective transport of cargo on RAB-5 and PI(3)P-positive early endosomes. This result suggests *C. elegans* WASH regulates sorting during endocytic recycling, through retrograde trafficking to the Golgi apparatus. (2) Using assays for embryonic development showed that loss of WASH components resulted in embryos that die during morphogenesis, and some that died earlier. Therefore, in contrast to loss of WASP or WAVE, which are needed during morphogenesis, loss of WASH resulted in some embryos that died before morphogenesis begins, due to cell cycle arrests. Beta-tubulin::GFP expression was strongly diminished in these embryos. It is known that the WASH complex assembles at Centrosomes, so that Centrosomes support WASH. We are therefore investigating if WASH feeds back on Centrosome function, to address the effect of WASH on microtubules (MTs). Thus, *C. elegans* has a conserved, 5-member WASH complex that appears to function, like its orthologs, in early endosome to Golgi retrograde transport. In addition, *C. elegans* provides a great system to investigate how the WASH complex coordinates MT-dependent events during early embryonic development.

Actin Cytoskeleton 1

P1013/B14

Unraveling the regulatory mechanism of FNBP4-mediated FMN1 regulation in actin cytoskeleton remodeling.

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Actin cytoskeleton is crucial for various cellular processes, such as cell motility, endocytosis, and DNA damage repair. These processes require extensive actin remodeling, which is regulated by numerous actin-binding proteins that control the assembly and disassembly of actin filaments. Formins, a bona fide group of actin cytoskeleton regulators, play a pivotal role in filament nucleation and elongation. In mammals, there are 15 formins, broadly classified based on their regulatory mechanisms into diaphanous-related formins (DRFs) and non-diaphanous-related formins. DRFs, such as mDia1 and Daam1, are regulated by Rho GTPase, releasing them from an auto-inhibited conformation. In contrast,

Formin1 (FMN1), a key member of the non-diaphanous formins, is not regulated by Rho GTPase, and the regulatory mechanisms for non-diaphanous formins remain largely uncharacterized. Therefore, we aimed to investigate the regulation of non-diaphanous formin FMN1. In our previous study, we identified an interaction between the N-terminal WW1-WW2 domains of FNBP4 (Formin-binding protein 4) and the C-terminal FH1-FH2 domains of FMN1. Surface plasmon resonance (SPR) and enzyme-linked immunosorbent assay (ELISA) demonstrated that the WW1 domain of FNBP4 specifically interacts with the FH1 domain of FMN1. Deletion of the WW1 domain (resulting in the N-ter ΔWW1 FNBP4 construct) abolished this interaction. Here, we aim to unravel the functional implications of the FMN1-FNBP4 interaction in actin cytoskeleton dynamics regulation. Using total internal reflection fluorescence (TIRF) microscopy and pyrene-actin polymerization assays, we found that FNBP4 inhibits FMN1-driven actin assembly in vitro. Furthermore, in the actin filament elongation assay, FNBP4 inhibits processive activity of FMN1, preventing it from displacing the capping protein CapZ at the growing barbed ends of actin filaments. We also found that the WW1-WW2 FNBP4 inhibits the bundling activity of the FH1-FH2 FMN1 in a concentration-dependent manner, while not affecting the bundling activity driven by the FH2 FMN1. In summary, we propose that FNBP4 functions as a stationary inhibitor of FMN1. This study can lead to the discovery of regulatory mechanisms specific to non-diaphanous formins, contributing to a deeper understanding of actin dynamics and providing new mechanistic insights.

P1014/B15

Rap1 and Rap2 GTPases Regulation and Function Through ARHGAP29 to Establish Cell Polarity and Promote Cell Migration.

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Cell migration is a dynamic and highly regulated process involving polarization of the migrating cell into a lagging and leading edge. These distinct edges are mainly established through the formation of branched actin networks at the leading edge and actin-myosin stress fibers at the lagging edge. We have focused on two members of the Ras family of GTPases, Rap1 and Rap2, as important regulators of cell polarity and the actin cytoskeleton. Both Raps display unique mechanisms of activation and localization regulation. Rap1 and Rap2 bind to a Rab40 E3 ubiquitin ligase complex which causes them to be tri-mono-ubiquitinated and activated. Ubiquitinated Rap2 is localized to the plasma membrane while non-ubiquitinated Rap2 is localized to lysosomes. Rap1 ubiquitination results in Rap1 localization to the cytoplasm while loss of Rap1 ubiquitination drives Rap1 localization away from the cytoplasm and to the plasma membrane. Interestingly, co-overexpression of both Rap1 and Rap2 results in Rap2 localization to lysosomes indicating that there is antagonism between Rap1 and Rap2 localization. To study the role of the Rap GTPases in actin regulation and cell migration, we made cell lines with either Rap1b loss or Rap2 loss. Interestingly, loss of Rap1b hyperpolarizes cells and impairs chemotactic migration. Loss of Rap2 disrupts cell polarity and prevents cells from migrating without a chemotactic stimulus. Further work in understanding how Raps are influencing the actin cytoskeleton and cell migration has suggested that they oppositely regulate lamellipodia localization of ARHGAP29, a putative RhoA GAP previously linked to regulating RhoA activity in cell-cell adhesions. ARHGAP29 has been linked to metastasis of multiple cancer types, however, how it functions in cell migration remains unclear. We have found that loss of ARHGAP29 abolishes cell polarity, resulting in cells with severely hindered random and chemotactic migration capabilities. Interestingly, these ARHGAP29 KO cells do not display major defects in dynamic ruffling actin, indicating that ARHGAP29 plays a role in localization of actin ruffling but not actin ruffling dynamics. These data suggest a mechanism by which Rap1 and Rap2 antagonistically

localize ARHGAP29 to the plasma membrane, allowing for ARHGAP29 activity as a RhoGAP to promote ruffling actin and thus polarize cells to form a lamellipodia.

P1015/B16

Cell-cell signaling makes an essential contribution to apical constriction by basolaterally recruiting Arp2/3 via Rac and WAVE.

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Apical constriction is a critical cell shape change that sculpts tissues into specific forms during development. How precisely-localized actomyosin regulators drive apical constriction remains poorly understood. *C. elegans* gastrulation provides a genetically and optically amenable model to address this question. The Arp2/3 complex has been shown to play essential roles during *C. elegans* gastrulation. To understand how Arp2/3 is locally regulated, we imaged embryos with endogenously-tagged Arp2/3 and nucleation-promoting factors (NPFs). The three *C. elegans* NPFs - WAVE, WASP, and WASH - showed distinct subcellular locations. Further, the NPFs colocalized with Arp2/3 and controlled Arp2/3 localization at each of these subcellular locations. These results indicate that three NPFs modulate Arp2/3 at different subcellular locations, which we have exploited to study the contributions of distinct populations of Arp2/3 in apical constriction. We found that WAVE depletion leads to highly penetrant gastrulation defects, WASP makes a fully redundant contribution, and WASH depletion did not cause noticeable defects. WAVE localizes basolaterally at the site of cell-cell contacts, and this localization is dependent on Rac (CED-10). We further found that establishing ectopic cell-cell contact between embryos is sufficient to recruit WAVE and Arp2/3, indicating that cells use cell-cell contacts as a symmetry-breaking cue for the localization of these proteins. We also noticed that CED-10, WAVE, and Arp2/3 depletion caused membrane blebs in early embryos. Because bleb formation depends on myosin activity, we asked if the gastrulation defects in WAVE-depleted embryos could be rescued by tuning myosin levels. We found that reducing myosin levels to 30-50% could restore normal membrane morphology and rescue the gastrulation defects in 40-70% of the embryos. Taken together, our results suggest that Rac signaling basolaterally, at cell-cell contacts, is essential for maintaining proper membrane-cortex linkage and contributes to the success of apical constriction.

P1016/B17

Actin-binding Protein as a Novel Damage-Associated Molecular Pattern.

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Damage-associated molecular patterns (DAMPs) are molecules known to be released from damaged or dying cells in response to stress. These molecules then activate the innate immune system, often by interacting with toll-like receptors (TLRs) present on immune cells. Although actin-binding protein profilin1 (Pfn1) is best known for its intracellular role as a regulator of actin cytoskeletal dynamics, we previously demonstrated that a small fraction of cellular Pfn1 is also externalized by various cells. However, what signals drive Pfn1 externalization and the biological significance of externalized Pfn1 remain unclear. In this study, we show that induction of genotoxic and endoplasmic reticulum (ER) stresses increases cellular Pfn1 mRNA expression and facilitates Pfn1 release in the extracellular milieu. We further show evidence for soluble Pfn1's binding to the cell membrane and induction of phenotypic changes when added in the extracellular space. Importantly, extracellular Pfn1 stimulates the expression

of pro-inflammatory cytokines/chemokines (IL1β, IL-6, and CXCL10) in macrophages in a dose-dependent manner and this effect is diminished significantly upon pharmacological inhibition of Myd88, a critical cofactor for TLR signaling. Collectively, these findings raise the possibility that cytoskeleton-regulatory proteins may be a novel class of DAMPs with downstream immunomodulatory action. This study could have a broad implication of extracellular Pfn1's role in the context of chronic inflammation for multiple disease types to be investigated in the future.

P1017/B18

TDP-43 Regulates Actin Dynamics.

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Amyotrophic lateral sclerosis (ALS) is an untreatable neurodegenerative disease where patients experience a persistent decline in motor functions. Trans Active Response (TAR) DNA-binding protein 43 (TDP-43) is implicated in almost all cases of ALS and to a lesser extent in several other neurodegenerative diseases. TDP-43 a biomolecular condensate-forming, multifunctional protein that performs diverse motor neuron cell functions including RNA metabolism, mitochondrial processes, and stress response which become disrupted in patients with ALS. While the actin cytoskeleton or its dynamics has not been directly connected to motor neuron dysfunction in ALS, it shares several essential cellular functions with TDP-43. Here we use biochemical assays with purified proteins to characterize a previously unknown direct interaction between TDP-43 and actin. We use total internal reflection fluorescence (TIRF) microscopy to visualize TDP-43 directly interacting with and altering in vitro actin polymerization, significantly reducing actin nucleation and slowing filament elongation. Therefore, TDP-43 partially inhibits actin polymerization.

P1018/B19

VopF is a processive polymerase of barbed and pointed ends of actin filaments.

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Actin is an essential protein required for force generation in key cellular processes including cytokinesis, cell migration, phagocytosis etc. Intracellular actin networks are thought to assemble by polymerization of filaments at their barbed ends and depolymerization at their pointed ends. This process, referred to as "treadmilling", forms the central bedrock of our current understanding of actin dynamics. Recent results from our lab challenge this fundamental paradigm. Using a combination of multicolor single-molecule and single filament experiments we have recently discovered the first ever processive polymerase that can elongate barbed as well as pointed ends. To elucidate the underlying mechanism of VopF-mediated polymerization, we propose a two state model where VopF can exist in a polymerization competent and a polymerization incompetent state. To test this model, we applied flow-induced pulling forces on filaments elongating from surface-anchored VopF molecules. Our results show that VopF's polymerization function is mechanosensitive i.e., its rate of polymerization increases under pN-range pulling forces which supports our two state model. Our new findings challenge the basic tenets of our current understanding of intracellular actin dynamics and call for reevaluation of previously established paradigms of actin assembly.

P1019/B20

Liquid-like Condensates that Bind Actin Drive Filament Polymerization and Bundling.

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The actin cytoskeleton forms filament networks that play a critical role in cell motility, endocytosis, and adhesion. The assembly of this network is facilitated by actin accessory proteins, which collectively determine filament network characteristics. Several cytoskeletal proteins have been shown to assemble into condensates via liquid-liquid phase separation, a process through which biomolecules self-assemble into a liquid-like condensed phase. Building on these findings, we recently showed that condensates consisting of the actin polymerase VASP can polymerize and bundle actin filaments. As actin polymerized inside VASP condensates, actin filaments accumulated at the inner surfaces of the condensates to minimize filament curvature. This partitioning led to the assembly of a peripheral, ring-like bundle of actin within condensates. When the rigidity of the actin ring overcame the surface tension of the VASP condensate, the filaments spontaneously straightened, deforming the initially spherical VASP condensates into rod-like structures filled with parallel bundles of actin filaments. Building on these findings, our ongoing work illustrates that actin polymerization and bundling by protein condensates do not require proteins with specific polymerase activity. Specifically, we show that condensates composed of Lamellipodin, a dimeric protein that binds actin but is not an actin polymerase, could also polymerize and bundle actin filaments. Additionally, we found that a monomeric form of Lamellipodin was also capable of forming condensates that polymerized and bundled actin. How does the formation of protein condensates confer this capacity upon Lamellipodin? Multi-valent binding to actin filaments is thought to underlie the function of canonical actin polymerases. Therefore, one possible explanation is that the condensate environment promotes multivalent interactions between Lamellipodin and actin. To investigate the potential contribution of protein condensates to actin bundling, we developed an agent-based model of filament rearrangement within spherical containers that mimic protein condensates. Guided by its predictions, we hypothesized that any condensate-forming protein that binds actin could bundle filaments. To test this idea, we added an actin-binding motif to Eps15, a condensate-forming protein that does not normally bind actin. The resulting chimera formed condensates that drove efficient actin polymerization and bundling. Collectively, these results demonstrate that filament polymerization and bundling are emergent properties of liquid-like protein condensates that bind actin, suggesting a general principle of actin organization through multivalent interactions.

P1020/B21

Capping Protein Mediates Competition among F-Actin Networks and Competes with Formin to Tune Network Architecture and Dynamics in the *C. elegans* Zygote.

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Eukaryotic cells assemble diverse filamentous (F-) actin-based networks, with distinct architectures and dynamics, to perform numerous essential cellular tasks. The properties of these highly specialized networks are specified by the activities of different actin binding proteins (ABPs) including actin assembly factors such as formin and the arp2/3 complex, bundlers, and regulators of actin filament

length, including Capping Protein (CP). Previous studies have established that competition among networks for a limited pool of actin monomers and ABPs helps coordinate the assembly of different F-actin networks. Here, we show that CP regulates this competition in the one cell *C. elegans* embryo (zygote). Additionally, within a network, CP competes with formin to limit actin filament elongation, thus regulating the initiation and maintenance of assembly of that network. Using *in vivo* multicolor near-TIRF microscopy, we simultaneously visualized formin, arp2/3 complex, and CP to characterize two F-actin networks in the zygote: filopodia and mini-comets. Filopodia are membrane-bound protrusions containing a shaft of bundled filaments assembled by formin and a base assembled by the arp2/3 complex, while mini-comets are assembled by the arp2/3 complex. We show that CP is dynamically enriched at the base of filopodia and throughout mini-comets. Depleting CP via RNAi increases the frequency and duration of filopodia assembly, while decreasing mini-comet assembly, suggesting that CP regulates the competition for resources between these two networks. The increase in filopodia assembly upon CP RNAi is reflected in an increase in initiation of filopodia and two-fold increase in filopodia lifetime.To understand the mechanisms that drive these changes, we used high-speed imaging to quantify the movements of formins along the shafts of filopodia in control and CP-depleted zygotes. We observe rapid movements of formins toward filopodia tips and slower movements away from the tips, suggesting that filopodia are maintained by a balance of initiation and termination of actin filament assembly in the filopodia shaft. In CP-depleted zygotes, the frequency of formin initiation is increased, suggesting that CP regulates initiation rates via competition with formin for binding barbed ends in the filopodia base, thus influencing filopodia lifetime. Together, these results reveal a role for CP in regulating the coordination between arp2/3 complex and formin to assemble and maintain dynamic F-actin based structures in the context of a crowded cytoplasm.

P1021/B22

TRIM9 Regulates Melanoma Adhesion, Actin Dynamics, and Motility.

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Cell shape change and motility involve remodeling of the actin cytoskeleton, cell adhesions, and plasma membrane. How these cytoskeletal and membrane remodeling are altered in pathological states remains largely unknown. Netrin is an extracellular morphogen which promotes neuronal morphogenesis and cancer progression. Here we examine the role of a brain enriched E3 ubiquitin ligase TRIM9 implicated in netrin dependent neuronal morphogenesis in the context of melanoma. We previously identified that TRIM9 regulates netrin dependent actin dynamics and exocytosis in developing neurons. Deletion of murine *Trim9* impairs neuronal migration, netrin induced axon turning, and axonal and dendritic branching, and increases exocytosis and filopodial stability. TRIM9 alters the dynamics of the actin polymerase VASP at filopodia tips via non degrative ubiquitination. TRIM9 is expressed in other motile cells, but the non neuronal role of TRIM9 is unknown. TRIM9 was identified as a potential prognostic biomarker in melanoma and high TRIM9 expression correlates with low patient survival. Melanomas undergo phenotype switching, where three distinct phenotypes exist that are associated with differential gene expression. Single cell RNAseq data from patient-derived melanoma indicate TRIM9 is highly expressed in phenotypes that correlate with poor prognosis. *Broadly our findings support the hypothesis that TRIM9 coordinates actin dynamics, adhesion, and exocytosis in melanoma to regulate cell motility and potentially invasion.* We show that in several human melanoma lines TRIM9 protein is enriched and netrin is secreted. Here we examine the role of TRIM9 in regulation of focal adhesions, exocytosis, migration, and invasion. Genetic loss of *TRIM9* increased random

migration velocity, but reduced directional persistence. We find that TRIM9 plays a role in promoting bleb like morphology and inhibits the ability of cells to durotax on soft shallow. Fluorescence recovery after photobleaching, Total internal reflection fluorescence and widefield microscopy revealed that loss of TRIM9 results in increased focal adhesions, cell size, and altered dynamics of focal adhesion proteins VASP, zyxin, and paxillin dynamics. TRIM9 knockout cells exhibit reduced filopodial length and density and altered filopodial localization of VASP and lamellipodial width. Zymography indicates that TRIM9 knockout cells display an increased degradative capacity. Current studies investigate how loss of TRIM9 alters parameters of cell contractility, exocytosis and *in vivo* metastasis to define the role of TRIM9 in melanoma motility. These findings suggest TRIM9 reduces adhesion and migration, increases proliferation and blebbing, and may be an important regulator of phenotype switching in melanoma.

P1022/B23

Isoform Specificity of a Compound Targeting Actin Filaments Containing Tropomyosin Tpms1.8 and 1.9.

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The unbranched actin filaments in mammalian cells are usually composed of co-polymers of a specific tropomyosin with actin. Microscopy and biochemistry techniques have demonstrated that the different tropomyosin isoforms are spatially segregated in a wide range of cells. Furthermore, genetic manipulation has revealed that the tropomyosins largely define the functional properties of actin filaments in an isoform-specific non-redundant manner. Hence, the development of compounds that target different tropomyosins are potentially valuable tools for cell biology as well as potential therapeutics. New generation compounds that target Tpm1.8/1.9, Tpm3.1/3.2 and Tpm4.2 have been developed and published. These compounds are all based on targeting differences in the N-terminus of these tropomyosins encoded by exon 1b. We have addressed the isoform specificity of the compound that targets the isoforms Tpm1.8 and Tpm1.9. These isoforms are identical except for an internal alternatively spliced exon. Tpm1.8/1.9 is primarily enriched in the lamellipodium of migrating cells but not in stress fibre bundles unlike Tpm3.1/3.2 and Tpm4.2 that are enriched in stress fibres. Exposure of fibroblasts and SK-N-SH cells to the compound Tpm189-3 (PubChem CID 18973468) results in dispersal of Tpm1.8/1.9 from the lamellipodium to a diffuse organisation in the cytoplasm. In contrast, at doses that disperse Tpm1.8/1.9, the compound has no impact on the association of either Tpm3.1/3.2 or Tpm4.2 with actin filament bundles. We also tested the impact of Tpm189-3 on cancer cell viability since compounds that target Tpm3.1/3.2 are highly cytotoxic to cancer cells. Tpm189-3 does not impact cancer cell viability at doses over 5-fold greater than the dose required to disperse the lamellipodial organisation of Tpm1.8/1.9. In addition, exposure of fibroblasts to Tpm189-3 at doses that disperse Tpm1.8/1.9 impact the polarity of these cells and lead to elongation with reduce width. Live imaging identifies a loss of persistent directional movement potentially as a result of disrupted cell polarity. We conclude that the amino acid sequence differences located at 7 positions in the first 16 residues of these isoforms provides sufficient specificity to generate compounds that target Tpm1.8/1.9 alone.

P1023/B24

Turnover Mechanisms of Nonmuscle Myosin IIA and IIB in Cells.

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Nonmuscle myosin II (NMII), essential for cellular contractility, comprises three isoforms—NMIIA, NMIIB, and NMIIC—which homo- and heteropolymerize to form NMII bipolar filaments. The specific interplay between these isoforms and their contributions to actin cytoskeletal organization remain unclear. We previously demonstrated that ectopic NMIIA expression in COS-7 cells, which lack NMIIA, redistributes NMIIB-dominated stress fibers. We hypothesized that NMIIA that is characterized by faster turnover of bipolar filaments, can dynamize the NMIIB filaments through copolymerization. However, the specific determinants of the NMIIA molecule responsible for its fast turnover are not fully defined. Here, we systematically analyzed the ability of NMIIA and NMIIB mutants to affect the NMIIB dynamics and distribution in COS-7 cells.
Using overexpression of the various forms of NMIIA and NMIIB that contained mutations expected to impair or enhance the turnover of these myosins, we showed that the NMIIA tail domain is a key driver of endogenous NMIIB redistribution and that both the presence of the NMIIA nonhelical tail (NHT) and phosphorylation of S1915 and/or S1916 are required to preserve the NMIIA's ability redistribute the endogenous NMIIB in COS-7 cells, whereas individual mutations (ΔNHT or S1915/1916/1943A) minimally impair the NMIIB distribution. In agreement, FRAP analysis revealed that the NMII versions capable of redistributing NMIIB exhibited faster recovery rates as compared with the constructs that failed to redistribute NMIIB. This underscores the importance of both the NHT and the C-terminal phosphorylation sites of NMIIA in regulating NMIIB distribution and suggests that a combination of these mechanisms allows NMIIA to exhibit fast filament turnover. Conversely, the "dynamizing" S1935D mutation in the NMIIB tail allows this mutant to redistribute endogenous wild type NMIIB, thus mimicking the effect of exogenous NMIIA. Collectively, our findings provide insights into how isoform-specific regulation of NMII filament turnover affects the organization and dynamics of the cellular contractile system.

P1024/B25

Actin filament assembly driven by surface-associated polymerases: a quantitative theory.

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Actin filaments created by the Arp2/3 complex form branched networks, whose growth can move and shape cellular membranes. Here, we show how membrane surfaces can locally accelerate filament assembly via clustering of proteins that bind actin monomers and/or profilin-actin complexes. Although effects on monomer orientation or 'steering' may enhance the rate of filament elongation, polymerase activity can be explained solely by clustering of polymerizable actin. Briefly, thermal fluctuations of the filament tip define a region of interaction with the membrane. Delivery of actin from solution to this region follows the formalism of Berg (1970), while delivery of actin to the growing filament is driven by two-dimensional diffusion of the filament tip across the membrane. We calculate the dependence of polymerase activity on both the surface density of actin-binding proteins and the solution concentration of polymerizable actin, and show that surface-mediated polymerization outpaces solution-mediated elongation, even at high concentrations of soluble actin (>100 μM). Finally, we expand our formalism to

describe the effects of barbed-end crowding, profilin dissociation, and compressive forces on surface mediated polymerization.

P1025/B26

A Role for WAVE Regulatory Complex in Bone Homeostasis and Osteoclast Function.

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Hem1 is the hematopoietic specific paralog of Nap1 and embodies an essential subunit of the WAVE regulatory complex (WRC), necessary for Rac1-mediated lamellipodial protrusion. We generated Hem1 knockout ($Hem1^{-/-}$) mice that develop an autoimmune disease (Salzer *et al.,* 2020) and concurrently display abnormal bone phenotypes reminiscent of osteopetrosis. Bone homeostasis is maintained by a balance between osteoblast and osteoclast activity. Osteoblasts descend from the mesenchymal lineage and express the paralogous Nap1, while osteoclasts originate from the hematopoietic monocytic/macrophage lineage, exclusively expressing Hem1. Our flow cytometry data reveal that $Hem1^{-/-}$ macrophages have a higher tendency to differentiate into osteoclasts compared to wild-type macrophages upon RANKL (Receptor Activator of Nuclear factor Kappa-β Ligand) stimulation. This is further corroborated by data obtained from live cell imaging uncovering that $Hem1^{-/-}$ macrophages differentiate faster into osteoclasts than their wild-type counterparts. *In vitro* studies also show that cell size and consequently the number of nuclei in osteoclasts differentiated from $Hem1^{-/-}$ bone marrow is significantly increased, indicating pronounced cell fusion. We additionally studied the expression of osteoclast-specific marker proteins such as RANK (receptor activator of nuclear factor-κB), or DC-STAMP (dendritic cell-specific transmembrane protein) in $Hem1^{-/-}$ and wild-type osteoclasts, uncovering an altered expression pattern at different stages of osteoclast differentiation, which is in line with altered differentiation. Moreover, $Hem1^{-/-}$ osteoclasts show a reduced ability to degrade bone matrix and immunofluorescence studies elucidate that this is accompanied by impaired ruffled border and discontinuous actin belt formation. We have shown that WRC deficiency causes an integrin activation defect in $Hem1^{-/-}$ macrophages (Stahnke *et al.,* 2021). To explore whether this also applies to osteoclasts and/or contributes to the phenotype, we probed the effects of selected extracellular matrices on osteoclast differentiation and found indeed significant differences. This study offers a new perspective on the role of Hem1 and in particular WRC in general on cell differentiation. In addition, these insights might aid the development of strategies to tackle bone related disease like osteopetrosis.

P1026/B27

A Sonic Hedgehog Deployment Complex Signals to Induce Cytoneme Formation.

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Embryonic development relies on intercellular communication via secreted morphogens. These signaling ligands facilitate pathway activation over long distances to instruct cell fate and pattern tissues. Sonic Hedgehog (SHH) morphogens play integral roles in a variety of developmental processes including neural tube patterning. SHH-producing cells extend long, actin-based “cytonemes” that facilitate direct transport of ligand to signal-receiving cells. However, these delicate signaling filopodia do not survive conventional fixation methods. To permit mechanistic studies on cytonemes in mammalian systems, our lab developed protocols preserving cytonemes for confocal analysis in cultured cells. Using these

techniques, we found that expression of SHH promotes cytoneme formation in Mouse Embryonic Fibroblasts (MEFs). We generated compound receptor knockout MEFs to determine the receptor requirements for SHH-stimulated cytoneme outgrowth. Based on these results, we describe here a novel SHH Deployment Complex (SDC), consisting ofSHH binding to DISP deployment receptor and at least one of the SHH coreceptors CDON or BOC. SHH binding to the SDC stimulates actin polymerization forcytoneme outgrowth. Since knockout of both BOC and CDON ablates SHH-induced cytonemes in MEFs, one of the primary functions of these coreceptors within theSDC is activation of cytoneme-initiating signaling downstream of SHH-coreceptor engagement. Using RNAscope *in situ* analysis at progressive embryonic time points, we found that CDON, not BOC, is expressed in the SHH-producing tissue of the developing mouse neural tube. Therefore, we conclude that DISP and CDON comprise the SDC in neural tube development. To better understand the mechanistic function of the SDC in cytoneme initiation signaling, we mapped the interaction between DISP and CDON and found that these receptors can interact through their intracellular domains. Binding of SHH to DISP causes conformational shifts in the C-terminal tail of DISP, alleviating this interaction and rendering CDON accessible for recruitment of actin-polymerization machinery. To assess the requirement for CDON in the generation of SHH-transporting cytonemes *in vivo*, we developed a protocol to preserve cellular extensions in the developing mouse neural tube for confocal analysis.

P1027/B28

Tensins are Regulated by Clusterin to Modulate Actin Dynamics and Extracellular Matrix.

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In our previous study, we demonstrated that the knockdown of clusterin, a secretory chaperone protein, enhanced actin polymerization with increasing cell adhesive interactions and extracellular matrix (ECM) levels and constitutive induction of clusterin expression decreased actin polymerization in trabecular meshwork (TM) cells. The TM tissue, found in the anterior segment of the eye, is composed of smooth muscle type of biomechanically sensitive cells, which aids in sensing and regulating the intraocular pressure. Actin cytoskeleton tension, cell adhesive interactions, and ECM stiffness play important roles in the pressure-sensing function of the TM. Therefore, this study aimed to elucidate the mechanistic evidence for clusterin gain-of-function on the actin and cell adhesive interactions in human TM (HTM) cells. To study this, we performed - A) tandem mass spectrometry-based global proteomic analysis on HTM cells constitutively expressing clusterin (AdCLU) compared to control (AdMT). Screening criteria protein level changes included $p \leq 0.05$ and mean$\pm 2\sigma$ of $\log2 \geq 0.3$ as the confidence fold change (FC). B) Label-free proteomic analysis of co-immunoprecipitated (Co-IP) samples with tensin 3 (TNS3) antibody to determine its interactors. C) The effect of siRNA-mediated loss of TNS3 (siTNS3), and the effect of pcDNA-EGFP-TNS3 (eTNS3)-mediated overexpression on actin fibers. D) Immunoblotting (IB) to analyze the effect of the siTNS3 on actin-associated proteins. Statistical analyses were performed using a student's t-test with significance at $p \leq 0.05$. Proteomic analysis showed AdCLU significantly reduced levels of actin alpha 1 ($p=0.046$, log2FC=-0.5), TNS1 ($p= 0.08$, log2FC=-0.23), TNS3 ($p=0.02$, log2FC=-0.44), and TNS4 ($p=0.04$, log2FC=-0.22) suggesting clusterin-mediated control of cytoskeleton and cell adhesive interactions. Confirmatory analysis by IB showed that AdCLU significantly reduced TNS3 ($p=0.01$). Proteomics of Co-IP samples using TNS3 antibody revealed interactions of TNS3 with major cytoskeletal and ECM proteins, including vimentin, actin, filamin-A, twinfilin-2, and tenascin-c. To better understand the functional role of TNS3, knocking down TNS3 using siTNS3 reduced F-actin distribution compared to siScramble (siScr). Further, IB analysis in siTNS3 compared to siScr showed a significant

downregulation of fibronectin (p=0.04) and vimentin (p=0.03). Contrarily, induction of TNS3 using TNS3 expression plasmid demonstrated increased F-actin and increased distribution of active integrin β1 and β3 compared to control. This novel study is a firsthand report on how clusterin regulates actin and the integrin-adhesome complex regulators like the tensins, which can modify fibrillogenesis via integrins and alter tissue biomechanics.

P1028/B29

Overexpression of Lifeact in the *C. elegans* body wall muscle causes sarcomere disorganization and embryonic or larval lethality.

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Live imaging of the actin cytoskeleton has been technically advanced by development of probes for actin filaments. Lifeact, a short peptide derived from yeast ABP140, is one of the most widely used F-actin probes. Although Lifeact is useful to label many actin-based structures in cells, several studies have shown that overexpression of Lifeact can be detrimental to normal biological processes in which actin plays a major role. Biochemical and structural studies have shown that Lifeact can slow down actin dynamics and compete with other proteins for F-actin binding. Here, I report effects of Lifeact on sarcomeric actin organization in the nematode *Caenorhabditis elegans* body wall muscle, which is an obliquely striated muscle with distinct sarcomeres. When mCherry::Lifeact was specifically expressed in the body wall muscle at low levels, it labeled sarcomeric actin filaments without noticeable abnormalities. However, when mCherry::Lifeact was overexpressed, actin filaments were severely disorganized into abnormal bundles in the muscle cells. Remarkably, worms carrying extrachromosomal arrays containing the transgene for muscle-specific expression of mCherry::Lifeact were ~40 % lethal either at late embryonic stages (~30 %) or the L1 larval stage (~10 %). Therefore, severe disturbance of the actin organization in the body wall muscle can cause developmental arrest of the whole animal. In contrast, RHS41, a strain carrying a single-copy transgene for muscle-specific expression of Lifeact::mRuby (Higuchi-Sanabria et al., 2018), was rarely lethal with a low-expression level of Lifeact::mRuby properly labeling sarcomeric actin filaments, indicating that this strain is suitable for live imaging of actin filaments in *C. elegans* muscle. In conclusion, overexpression of Lifeact in *C. elegans* can cause severely detrimental effects on actin organization, and expression levels of Lifeact need to be kept at low levels to avoid artificial effects.

P1029/B30

Dynamic PIKfyve-containing puncta regulate lamellipodia dynamics.

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Cell migration is an essential phenomenon in diverse biological processes including embryonic development, immune responses, and tissue repair. This directed movement of cells is controlled by chemical cues and signaling pathways. The force generated by actin polymerization within the cells drives cell migration by projecting membrane protrusions, including lamellipodia, at the leading edge of cells. Among those protrusions, lamellipodia are the major driver of cell migration in various cell types and are controlled by actin dynamics. Importantly, we and others recently reported that the PI3P 5-kinase, PIKfyve, may also plays a role in cell migration. This result was surprising because PIKfyve was

best known for roles in the regulation of lysosomes, and in autophagic degradation. We showed that PIKfyve contributes to cell migration in part via an unexpected role in recycling β1-integrin from early endosomes to the plasma membrane. Our new unpublished data suggests that PIKfyve has additional critical roles in cell migration via the regulation of actin dynamics in lamellipodia. Notably, we observed that acute chemical inhibition of PIKfyve for 5-10 minutes impaired lamellipodia dynamics and reduced the levels of F-actin in cell protrusions. Using TIRF live-cell imaging, we showed that a pool of dynamic PIKfyve-containing puncta localize to the cell protrusions. To gain insight into downstream targets of PIKfyve, we performed proximity-dependent biotinylation and we are currently pursuing targets related to actin dynamics. Together our new studies expand the known roles for PIKfyve, which was formerly thought to solely govern endo/lysosomes, and importantly suggest that PIKfyve is a regulator of multiple F-actin structures and cell migration.

P1030/B31

The Roles of Arp2/3 Complex Nucleation Promoting Factors in the C. elegans Single-Cell Embryo.

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The Arp2/3 complex is the only known actin assembly factor capable of forming branched filamentous actin (F-actin) networks. In the single-cell C. elegans embryo, Arp2/3 complex concurrently assembles multiple branched F-actin networks at the cell cortex: punctate networks called mini-comets, and the bases of filopodia. Mini-comets are entirely branched, while filopodia are hybrid structures with a branched base and a bundled tip elongated by the formin Cyk-1. To investigate whether Arp2/3 complex was differentially activated in filopodia bases and mini-comets, we sought to characterize the activity of Wiskott-Aldrich Syndrome Protein (WASP) and WASP family verprolin-homologous protein (WAVE) complex, two well known branched actin nucleation promoting factors (NPFs) which are expressed at the single-cell stage.

Using two and three color live cell TIRF microscopy, we observed that both WAVE and WASP are enriched at the anterior cortex, but with distinct localization patterns. While WASP localizes to all cortical branched F-actin networks, WAVE colocalizes with Cyk-1 to the tips of filopodia rather than the branched base. We also observed that RNAi mediated knockdown of WAVE significantly reduced filopodia assembly. Filopodia numbers were not reduced in WASP mutants or when Arp2/3 complex is knocked down, however both perturbations result in altered filopodial dynamics.

These results suggest that WAVE and WASP are working together, and with other actin binding proteins, to assemble filopodia with the proper dynamics and spatiotemporal localization. However, whereas WASP is likely acting to promote Arp2/3 mediated branched actin assembly in the base, WAVE is likely acting in filopodia initiation independently of Arp2/3 complex. Specifically, WAVE could be acting directly on actin filaments as a processive elongator or collaboratively with another actin assembly factor at filopodia tips.

P1031/B32

System Integration through Contractility Kits Mediate Cell Shapes Change Processes.

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Our comprehension of biological systems often rests within isolated domains, for example, with the cytoskeleton conventionally recognized for its structural and contractile roles in cellular processes, like

cytokinesis, motility, and morphology. In *Dictyostelium discoideum*, the presence of pre-assembled contractility kits (CKs) in the cytoplasm highlights a nuanced aspect of cytoskeletal function. These CKs, comprising various cytoskeletal components, respond to mechanical stress by accumulating along the cortex. Recent investigations in our lab, including genetic suppressor selections and proteomic analyses, have illuminated the cytoskeleton's involvement in intersystem crosstalk. Two proteins, RNA-binding protein 1 (RNP1A) and methylmalonate-semialdehyde dehydrogenase (mmsdh), emerged as potential mediators of intersystem communication with the cytoskeleton. RNP1A contributes to cell mechanics and mechanoresponsiveness while also binding to several mRNAs that encode proteins involved in macropinocytosis. Manipulation of RNP1A levels alters macropinocytosis activity, highlighting its role in this process. Proteomic studies reveal RNP1A's interaction with cortexillin I and IQGAP1, key CK components, indicating its involvement in cytoskeletal dynamics. On the other hand, mmsdh, an enzyme involved in valine catabolism, exhibits localization within mitochondria and the cytoplasm. In addition to its metabolic functions, mmsdh's role in cellular processes remains poorly understood. Notably, overexpression of mmsdh rescues cytokinetic defects caused by phosphomimetic non-muscle myosin II mutations. Proteomic analyses identify mmsdh's interaction with cortexillin I and myosin II, further implicating its involvement in CK dynamics. Knocking down mmsdh expression introduced several phenotypes such as a slower growth rate, reduction in cell size, and aberrant cytokinetic behavior. Combining insights from both studies, we formulate two hypotheses: first, RNP1A orchestrates coordination between macropinocytosis and the cytoskeleton through interaction with CK components; second, mmsdh modulates cytoskeletal dynamics and metabolic function via the CK network.

P1032/B33

Determining how physiological profilin-actin concentrations affect G-actin partitioning between Arp2/3 complex- and formin-mediated F-actin network assembly in fission yeast.

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Actin-binding proteins (ABPs) regulate filamentous actin (F-actin) elongation, architecture, and stability, which facilitate the formation of distinct F-actin networks for diverse fundamental cellular processes. The actin assembly factors formin and Arp2/3 complex compete for a limiting globular actin (G-actin) pool, which temporally controls F-actin network assembly by incorporating G-actin into different F-actin networks. The small ABP profilin regulates G-actin competition by favoring G-actin partitioning into formin- over Arp2/3 complex-mediated networks. Profilin binds most of the G-actin pool to maintain an unpolymerized G-actin population and partitions G-actin in fission yeast to accelerate formin-mediated F-actin elongation but inhibits Arp2/3 complex-mediated F-actin branching. I am using in vitro spontaneous actin assembly assays with fission yeast formins, Arp2/3 complex, and profilin-actin visualized with Total Internal Reflection Fluorescence Microscopy (TIRFM) to determine how profilin affects competition between Arp2/3 complex and formin for G-actin as well as how physiological profilin-actin concentrations affect formin-mediated F-actin elongation and Arp2/3 complex-mediated F-actin branching. Understanding whether profilin affects G-actin partitioning at physiological concentrations in vitro and the profilin’s Arp2/3 complex inhibitory mechanism is critical to understanding how profilin can exert spatial-temporal control of F-actin network assembly.

P1033/B34

Emergent Mechanisms of Load Adaptation in Endocytic Actin Networks From Force-Attenuated Capping.

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Force production by actin polymerization is crucial for several membrane-deforming cellular processes. These forces can be explained by an elastic Brownian Ratchet mechanism: actin filaments growing against a load fluctuate due to thermal motion, opening gaps for monomer addition, and restoration of filament position pushes the load forward. Critically, this implies that the rate of filament capping decreases with antagonistic force experienced by the filament (force-attenuated capping), which governs the load adaptation of in vitro actin networks by increasing filament density (Li et al., 2022). However, the mechanism by which such single filament load-dependent rates influence load adaptation by assemblies of actin networks participating in cellular processes remains unexplored. To address this question, we extended our experimentally-grounded agent-based model of actin networks at sites of clathrin mediated endocytosis (Akamatsu et al., 2020) to include force-attenuated capping, constrained by published in vitro measurements. We hypothesized that force-attenuated capping would allow endocytic actin filaments to adapt to increased loads by growing longer and thereby produce more force. Indeed, we found that implementing force-attenuated capping in the model allowed actin networks to produce ~2x more cumulative force and further internalize simulated endocytic pits. To investigate the mechanism by which force-attenuated capping increased endocytic pit internalization, we quantified filament lengths and numbers. Surprisingly, force-attenuated capping led to only a small (<10%) increase in average actin filament lengths. This is likely because only a small fraction of filaments experienced force, a previously unappreciated feature of such actin networks. In contrast, force-attenuated capping led to a ~40% increase in the total number of actin filaments, indicating an emergent consequence of increased filament nucleation. Importantly, under increasing load from membrane tension, force-attenuated capping led to further increased force production and more nucleated actin filaments. Taken together, these results identify a novel form of load adaptation, by which attenuating the capping rate of a small number of actin filaments leads to disproportionately increased filament nucleation, to increase force production against increasing resistance in endocytosis.

P1034/B35

Low intensity vibration improves apparent cell density and F-actin modeling in a 3D micro pellet model of chondrogenesis.

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Loss of mechanical loads during the transition from cartilage to bone, as it happens on developmental dysplasia of the hip, are the leading cause of early-onset hip osteoarthritis in individuals under the age of 50 among Americans. Previous studies have shown that low-intensity vibrations (LIV) enhance bone formation in both animals and in vitro models, as it upregulates pathways involved on chondrogenic and osteogenic responses, facilitating tissue differentiation. However, no studies have specifically explored the role of LIV in regulating chondrogenic tissue development. This study aims to address this gap by investigating the effects of LIV on the chondrogenic differentiation of MSCs using a hydrogel model, focusing on LIV's potential to enhance differentiation. To investigate, we encapsulated micro-pellets of MSCs ($6x10^4$ cells/pellet) in a hydrogel, with approximately $9.6x10^6$ cells/hydrogel. The samples were

divided into four groups: +LIV and -LIV, both with and without chondrogenic medium subgroups. For the +LIV groups, the gels were vibrated (25Hz, .5g) 4x/day for 14 days. Upon harvesting, we assessed cell viability, cell and actin density, and glycosaminoglycan (GAG) production using standard IHC staining protocols. Our results indicate that combining LIV with chondrogenic medium improves cell viability (+41%), cell density (+14%), and F-actin density (+74%). When pellets from the non-chondrogenic medium group were exposed to LIV, even greater increases were observed: +70% in cell viability, +103% in cell density and +1096% in fiber density. We did not observe significant changes in GAG with LIV. These results strengthen the idea that mechanical strains provided by LIV might help maintain cytoskeletal integrity, which in turn supports cell survival and differentiation. This study highlights the potential of LIV to enhance chondrogenic differentiation and improve protocol viability using MSCs. It also opens new avenues for exploring how mechanical strains influence the regulation of the cytoskeleton, and key markers in both chondrogenic and osteogenic pathways. As β-catenin (1) plays a pivotal role in chondrocyte development (maturation, hypertrophy, organization, and plasticity) and the formation of primary ossification centers, and (2) has shown to be responsive (upregulated) by LIV in the past, we are currently investigating the role of LIV in regulating β-catenin in chondrogenic tissues.

P1035/B36

Bequeathing a fair inheritance:How an unconventional fungus divides its growth potential among multiple daughter cells.

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Tractable yeast model systems have propelled fundamental discoveries in cell biology, but they do not encompass the full diversity of fungal cell biology. Unlike well-studied yeasts that produce only a single daughter cell (bud) per cell cycle, the unconventional yeast, *Aureobasidium pullulans*, can produce multiple buds simultaneously in a single cell cycle. This growth strategy raises the question of how growth potential is distributed between a mother's multiple offspring. In *S. cerevisiae*, post-Golgi secretory vesicles produced in the mother carry new cell membrane and cell wall components to the bud along polarized actin cables. We find that *A. pullulans* similarly produces secretory vesicles in the mother cell. These vesicles appear to be delivered to each bud at similar rates, because sister buds growing from the same mother grow at similar rates (CV = 0.15). Interestingly, buds grow at similar rates even in cases where the bud sites are asymmetrically distributed around the mother. Modeling suggests that in such situations, clustered buds would compete for local vesicles while distant buds would be able to access vesicles from a larger mother volume. This raises the question of how vesicle traffic to each bud is equalized. *A. pullulans* formins localize to each bud tip and build a polarized and dynamic network of actin cables that extends along the mother cell cortex. However, the network does not exhibit obvious compensation that could explain equal vesicle delivery to buds in differing geometries. Additionally, *A. pullulans* develops an interphase microtubule network that extends throughout the volume of the mother cell with the plus ends of microtubules oriented into each bud. By tracking processive vesicle movements in live cells, we find that both F-actin and microtubules can mediate vesicle transport. Based on these findings, we speculate that each bud's actin network catches and delivers vesicles from the bud-proximal mother volume, while microtubule networks may help to distribute vesicles throughout the mother so that each bud's actin network can access similar numbers of vesicles.

P1036/B37

Actin Modification by Mical and SelR Is Necessary for Actomyosin Ring Dynamics and Efficient Cell Wound Repair.

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Individual cells within tissues and organs are subjected to damage caused by daily wear-and-tear and environmental/physiological stresses. To survive this damage and remain functional, cells have a robust repair mechanism comprised of rapid membrane resealing/remodeling and dynamic cytoskeletal repair at the cell cortex that are initiated by calcium influx. Actomyosin ring contraction at the wound edge is one mechanism that can generate the physical force needed for wound closure. However, little is known about how the actomyosin ring assembles upon wounding, is dynamically organized, or disassembles after wound closure. Using the *Drosophila* cell wound repair model, we recently found that Rab35 is recruited to wounds and its RNAi knockdown disrupts actomyosin ring assembly and disassembly and exhibits slow wound closure. Rab35, a member of Rab family GTPases, regulates membrane trafficking and cytoskeleton dynamics in many cellular processes. In particular, Rab35 regulates Mical, a redox enzyme that binds to F-actin and promotes F-actin clearance during cytokinetic abscission. SelR is a methionine sulfoxide reduction enzyme that works as a counterpart to Mical. We find that both Mical and SelR are recruited to cell wounds. We find that both Mical and SelR RNAi knockdowns exhibit disrupted actomyosin ring organization and dynamics at the wound edge. To further examine the redox reaction of actin during cell wound repair, we generated a transgenic line having a point mutation on Methionine 44 that is known to be oxidized by Mical. Consistent with Mical knockdown, the M44 point mutation impairs actomyosin ring formation leading to inefficient cell wound repair. Our results suggest that actin modifications play a mechanistic role in the assembly and disassembly of the actomyosin ring, which is necessary for efficient cell wound repair.

P1037/B38

Identification of TPD52L2 as a Regulator of Actin Dynamics and Cellular Mechanoadaptability.

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Tumor protein D52 like 2 (TPD52L2) is part of the TPD52 protein family and is known to be overexpressed in a variety of malignant diseases. This includes breast cancer, where its overexpression correlates with a reduced overall survival. Recently, TPD52L2 was reported to be associated with intracellular nanovesicles, indicating its involvement in various membrane trafficking pathways. Our study unravels the role of TPD52L2 as a regulator of the actin cytoskeleton in breast cancer cells. Depletion of TPD52L2 results in a strong reduction of stress fibers, altered dynamics of actin protrusions and finally a thickening of the cortical actin layer. Mechanistically, we demonstrate that TPD52L2 interacts with Cullin-5, a central component of the Cullin-RING ubiquitin ligase (CRL) complex. Cullin-5 functions as a scaffold for the recruitment of Rab40 family members, which act as substrate adaptors for the ubiquitination and degradation of cytoskeletal proteins. The actin phenotype induced by silencing of TPD52L2 was partially rescued by co-depletion of Rab40c. We are currently testing a model wherein TPD52L2 regulates Rab40c-mediated degradation of actin regulators. As a result of the increased cortical actin in TPD52L2 depleted cells, aberrations of the mechanical properties were observed. Atomic force

microscopy measurements confirmed an increase in the rigidity of affected cells. Therefore, we determined whether TPD52L2 regulates cellular mechano-adaptability. We found that silencing TPD52L2 reduced the spreading area on hydrogels with varying substrate rigidities. Furthermore, a significant reduction in migratory speed was observed in a 3D random migration assay. Both, cell spreading and the migration defect were rescued by low-dose treatment with a ROCK-inhibitor, which reversed the effects of TPD52L2-loss on cellular rigidity. Altogether, our results imply TPD52L2 as a coordinator of cellular rigidity via its regulatory capacities on the actin cytoskeleton.

P1038/B39

Endothelial Cytoskeletal and Nuclear Dynamics During Sprouting Angiogenesis in Progeria.

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Hutchinson-Gilford Progeria Syndrome (Progeria) is an accelerated aging disorder caused by a *de novo*heterozygous mutation of Lamin-A, which results in an accumulation of a splicing isoform, termed Progerin, that destabilizes the nucleoskeleton. Due to the expression pattern of Lamin-A, the vasculature is highly susceptible to Progeria and death of children with Progeria is largely due to cardiovascular disease. Here, we tested the hypothesis that destabilization of the nuclear lamina by Progerin alters the nucleoskeletal-cytoskeletal interface and downstream cytoskeletal dynamics of endothelial cells during sprouting angiogenesis. Progeria mice (LMNA G608G) showed delayed retinal vasculature development with reduced endothelial cell migration at 6-days old. By 20 weeks, Progeria mice have significantly more junctional defects in the aorta endothelium, impaired endothelial migration in wound healing and significant smooth-muscle loss. While these findings are well characterized, the cell autonomous nature of endothelial defects in Progeria remains poorly understood. Therefore, we isolated brain endothelial cells from 6-day old Progeria mice and grew vessels *ex vivo*. Compared with wildtype control vessels, the Progeria vessels grew smaller with reduced lumen hollowing. This result was reproduced in HUVECs mosaically transduced with GFP-Progerin. Nuclei in healthy endothelial cells reshape during vessel hollowing, yet live imaging of GFP-Progerin nuclei revealed large or misshapen nuclei that failed to relocate during lumenogenesis. GFP-Progerin nuclei were significantly less likely to migrate into a vessel sprout and vessels containing a GFP-Progerin cell grew less compared to those without. These results led us to investigate the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex. Indeed, disruption of the LINC complex resulted in vessel sprouts phenotypically similar to GFP-Progerin vessels, however with less severity. This data suggests that interface impairment from Progerin results in changes of the integrity of the actin cytoskeleton and uncoordinated nuclear movement during sprouting angiogenesis.

P1039/B40

Coronin 1A and TRIM67 collaborate in netrin-dependent neuronal morphogenesis.

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Neurons progress through several developmental stages during the establishment of neuronal circuitry. The dramatic neuronal shape changes during these morphological events depend on the cytoskeleton remodeling machinery. Here we are describing a novel role for the actin binding protein Coronin 1A (Coro1A) in regulating neuronal morphogenesis in a TRIM67-dependent fashion. Previously, our lab discovered that the E3 ubiquitin ligase TRIM67 is highly enriched in the developing cortex and is

essential for appropriate neuronal morphogenesis. TRIM67 localizes to growth cone and actin-rich filopodia structures. Interestingly, neurons lacking TRIM67 exhibit aberrant axonal growth cone morphology and defects in netrin-dependent axon turning and branching, but the underlying molecular mechanisms by which TRIM67 regulated neuronal morphogenesis was unknown. As E3 ubiquitin ligases typically have multiple substrates, we conducted an unbiased proximity-dependent biotin identification assay to identify putative TRIM67 substrates. This identified Coro1A as a potential TRIM67 binding partner. Coro1A is a conserved F-actin binding protein crucial for actin dynamics, yet its role in brain development remains elusive. We validated that Coro1A and TRIM67 are interacting partners. Combining computational structural/docking analysis with biochemistry assays, we show that the C-terminal coiled-coil domain of Coro1A is essential for Coro1A:TRIM67 interaction. We demonstrate that Coro1A protein level increases during neuronal maturation in vitro and in vivo. Through TIRF microscopy and immunofluorescence staining, we find that Coro1A is enriched in growth cones of developing cortical neurons and localizes to the base of filopodia structures, proximal to the cofilin-coated actin bundles known as cofilactin. We found loss of Coro1A or TRIM67 increases the length of cofilactin. Using cultured cortical neurons from *Coro1a*$^{+/+}$ and *Coro1a*$^{-/-}$ littermates, we show that Coro1A reduces growth cone size and is required for netrin-dependent axon turning and branching, similar to TRIM67. Additionally, by performing rescue experiments we show that Coro1A:TRIM67 interaction is important for netrin-dependent changes in growth cone area and axon branching. Finally, we demonstrate that Coro1A is ubiquitinated by immunoprecipitating Coro1A under denaturing conditions. Ongoing work is investigating whether Coro1A regulates netrin-sensitive corpus callosum morphology. *These findings suggest a novel role for Coro1A in regulating netrin-dependent neuronal morphogenesis, likely functioning downstream of TRIM67.*

P1040/B41

Twinfilin, formin, and capping protein form a multicomponent complex at the actin barbed end.

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Actin cytoskeletal dynamics are crucial for key processes like embryonic development, neuro-degenerative diseases, and cancer metastasis. However, our understanding of the underlying molecular mechanisms remains incomplete. Actin elongators (formin), blockers (capping protein), and depolymerases (twinfilin) all function within a shared cytoplasm. On their own, these proteins display distinct activities, but how they work together in the cell is unclear. Using microfluidic assisted-total internal reflection fluorescence microscopy, we found that polymerases, blockers, and depolymerases can simultaneously bind the same filament barbed end to influence actin assembly. Simultaneous presence of these proteins at the barbed end leads to faster protein transitions and tuning of elongation rate as well as filament length. Multispectral single-molecule experiments reveal that twinfilin can bind formin-bound barbed ends only in presence of capping protein. The ternary complex between the three proteins is short-lived and results in rapid dissociation of CP from the barbed end, leaving behind a formin-bound, quickly elongating filament. Our findings thus suggest that the depolymerase, twinfilin, might function as a pro-polymerization factor when all three proteins are present. Taken together, our findings can potentially explain the wide diversity in size and dynamics of cellular actin structures *in vivo*.

P1041/B42

Cortical continuity of actin cables requires microtubules and the dynamic maintenance of actin filaments.

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Stable actin structures like microvilli and stereocilia exhibit dynamic maintenance, the balanced addition and subtraction of monomers in established filaments. We have uncovered a role for dynamic maintenance in the persistent cortical association of actin cables and a potential role for microtubules (MTs) in the regulation of dynamic maintenance. In late oogenesis, a syncytial network of nurse cells rapidly expels its cytoplasm into the oocyte through ring canals ("dumping"). Prior to dumping, actin cables initiate at the cortex and elongate toward the nucleus in each nurse cell. These cortically-associated actin cables push the nuclei away from the ring canals to prevent obstruction during dumping. Villin and Fascin produce the bundles of parallel actin filaments that comprise these cables. We showed that cable initiation and elongation are controlled by the collaboration of Enabled (Ena) and Diaphanous (Dia), and require a co-aligning, stable MT network. We tested the roles of MT regulators, including γ-tubulin and CLIP-190, essential for establishing the MT network. Surprisingly, the majority of actin cables produced in the absence of robust MT networks were not cortically associated but instead were “free”. These free cables initiated cortically, lost their cortical continuity, and shortened in the cytoplasm. MT stabilizing taxol produced free cables that elongated instead. These results suggest that MTs control the cortical association of actin cables, and the growth or shrinkage of actin filaments within the cables. There is no known mechanism for cortical tethering of these actin cables. To determine how MTs contribute to the cortical continuity of actin cables and modulate their growth, we tested the potential roles of filament elongation rate by Dia/Ena and bundling by Villin. None of these manipulations produced significant numbers of free cables despite exhibiting some defects in the MT network. Because free cables either shrank or grew depending on the MT manipulation, we tested the hypothesis that dynamic maintenance, downstream of MT function, enables cortical continuity. Remarkably, favoring filament assembly by reducing Cofilin activity produced free cables that grew in a formin-dependent manner while favoring filament disassembly with latrunculin produced shrinking free cables. Preliminarily, neither change to dynamic maintenance resulted in apparent defects in the MT network. Taken together, our findings suggest a model in which MTs regulate the dynamic maintenance of actin filaments, and that dynamic maintenance of filaments is required for the cortical continuity of the actin cables in which they reside.

P1042/B43

Animal septins can contain functional transmembrane domains.

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Septins, a conserved family of filament-forming proteins, contribute to eukaryotic cell division, polarity, and membrane trafficking by scaffolding other proteins to cellular membranes. The mechanisms by which septins associate with membranes are not well understood but can involve three polybasic domains and/or an amphipathic helix. We leveraged the nematode *Caenorhabditis elegans* as a model organism to uncover motifs involved in septin recruitment to membranes because of its small number of septin gene products. The *C. elegans* genome possesses only two septin genes: *unc-59*, encoding the SEPT7-like UNC-59 protein, and *unc-61*, encoding the SEPT6-like protein UNC-61. Here, we report

identifying an isoform of UNC-61 (UNC-61a) that is predicted to contain an N-terminal transmembrane domain (TMD). The predicted TMD sequence is sufficient to drive an otherwise cytoplasmic probe to cellular membranes in intact cells. The TMD isoform is expressed in a subset of the tissues where the previously known septins were implicated to act: the egg-laying apparatus (vulva and uterus) and the pharynx. Animals lacking expression of the TMD-septin had a protruded vulva that remained in an abnormal, open confirmation. The UNC-61a null animal was phenocopied by deleting only the TMD, suggesting that the TMD is required for the function of UNC-61a. Given the conservation of sequence and function of septins, we reasoned that TMD-septins could be found throughout opisthokont phylogeny and we confirmed via a bioinformatics survey using UniProt. None of these TMD-septins had been previously recognized or studied, although TMD-septins were predicted to exist in some non-opisthokont organisms, including the unicellular alga *C. reinhardtii* and the ciliate *T. thermophila*. Notably, the TMD was a feature of some SEPT6 family members in some mammals. We demonstrated that the TMD-containing sequence of a primate TMD-septin was sufficient for localization of an inert probe to cellular membranes. Addition of the TMD-containing sequence from a non-model primate to its human homolog led the latter to relocalize from actin-based structures to membranes. Together, our findings reveal a novel mechanism of septin-membrane association with profound implications for the dynamics and regulation of this association.

P1043/B44

Structural Changes in Actin Filaments Induced by Mechanical Force Stimulation.

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Actin molecule is composed of large and small domains separated by an ATP-binding cleft. Previous studies have suggested that relative twisting between these domains is related to changes in the affinity of actin-binding proteins (ABPs). Additionally, it has been proposed that actin undergoes structural changes in response to external forces. These findings imply that actin may sense mechanical forces and undergo conformational changes that modulate its affinity for ABPs in response to tension. In other words, actin could function as a mechanosensor, capable of converting mechanical forces into intracellular signals. The purpose of this study is to provide evidence supporting the hypothesized relationship between force, the molecular structure of actin, and ABPs interactions. Specifically, we aim to demonstrate that actin undergoes a structural change when subjected to tension and that its role as a mechanosensor is attributed to these structural changes. To detect structural changes in actin, we utilized FRET-labeled actin, in which fluorescent dyes are attached to the tips of large and small domains of actin. For the experiments, F-actin was prepared by copolymerizing biotin-labeled actin with FRET actin. The F-actin was then trapped between the beads and a microneedle via biotin-avidin interaction to apply tension. Structural changes in actin were monitored by observing change in FRET intensity when tension was applied through microneedle manipulation. The results indicated that the FRET intensity (acceptor/donor) for most F-actin decreased by 5%~10% following tension application, compared to pre-tension levels, and returned FRET intensity after the F-actin was broken. This suggests that the applied tension induced a structural change in the actin, increasing the distance between the donor and acceptor fluorophores, resulting in decreased FRET intensity. The subsequent recovery of FRET intensity upon F-actin breakage suggests a relaxation of the induced torsion. These findings provide evidence that actin undergoes tension-induced conformational changes, supporting the proposed relationship between mechanical force, actin structure, and ABP interactions.

Intracellular Transport: Molecular Motors

P1044/B45

Dynein activity during phagocytosis and Golgi positioning is regulated differentially by isoforms of the adaptor ninein in macrophages.

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Cytoplasmic dynein is an ATP-driven motor complex that plays a critical role in force generation to position organelles and translocate various cargo along the microtubule (MT) cytoskeleton. Dynein recruitment, regulation of pulling forces, and cargo specificity are regulated by specialized sets of cargo-adaptors and motility activators. In macrophages, the innate immune cells, the binding of foreign particles to the cell surface induces a robust formation of elaborate pseudopods known as phagocytic cups that extend out and around the target particle that is followed by inward translocation of the membrane-bound particle within 10 minuets of contact. While forces produced by F-actin and myosin motors have been implicated in phagocytosis, it is unclear whether dynein and cargo-adaptors contribute to the earlier steps of particle internalization and phagosome formation. Here, we reveal that ninein, a MT minus-end-associated protein that localizes to the centrosome, is also present at the phagocytic cup in macrophages. Ninein forms membrane-bound clusters on phagocytic cups that do not nucleate non-centrosomal MTs but instead mediate the assembly of the dynein-dynactin complex at active phagocytic membranes within the first 4 minutes of particle binding. Both ninein depletion and pharmacological inhibition of dynein activity by Dynarrestin reduced the inward displacement of the forming phagosome into the macrophage delaying the downstream process of phagolysosome formation. Furthermore, using quantitative RT-PCR, we identified two isoforms of ninein expressed in macrophages: a canonical ninein^{CAN} isoform that contributes to nearly 80% of all the ninein mRNA pool, compared to the other isoform, ninein^{Isoform2}. We found the presence of either isoform rescued the inward displacement defect observed by total ninein depletion, suggesting redundancy in regulating dynein recruitment/ inward pulling of the nascent phagosome. Consistent with conserved interactions with dynein, depletion of either isoform reduced dynein/dynactin at the MTOC to similar levels. Interestingly, we found that the presence of the ninein^{CAN} but not ninein^{Isoform2} rescued the severe morphological and positioning defects in Golgi observed in total ninein depletion. Defects in Golgi in cells lacking ninein^{CAN} were not due to reduced MTs, since depletion of ninein^{CAN} and ninein^{Isoform2} had normal MTs growing from the MTOC. Taken together, our analysis shows that ninein is key in regulating dynein activities and highlight the utilization of isoforms of cargo-adaptors as a potential mechanism for regulating cargo specificity.

P1045/B46

A Weak 5'-Splice Site Causes Exon Skipping of a Novel Myh9 Isoform, NMII A2.

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The Non-muscle myosin IIA, NMIIA is one of the major motor proteins responsible for generating contractile forces necessary for various cellular processes such as cell adhesion, migration, membrane trafficking and cytokinesis. Recently, we discovered that alternative splicing at loop2 region of its heavy

chain (NMHC IIA, encoded by Myh9 gene) pre-mRNA generates a novel motor inactive isoform, named NMIIA2, which was found to be expressed only in brain tissues. This raised the question, what factors promote the inclusion of 63-nucleotides (A2-exon) in neuronal cells. Here, we report that A2-exon is a cassette type-exon between two constitutive exons. Using systemic deletion of flanking intronic sequences, incorporating mutations at the 5' splice site and branch-point sequences, we establish that the skipping of the A2-exon arises due to the presence of a weak 5' splice site of A2 exon. On the contrary, the trans-regulatory brain tissue-enriched splicing factor, RbFox-3, blocks the skipping of A2-exon through its association with the weak 5' splice site. Increasing the strength of the 5' splice site further blocks the skipping of A2-exon. Altogether, these data suggest that interaction between the weak 5' splice site of A2-exon and RbFox-3 regulates the inclusion of A2 insert at loop 2 region of NMHC IIA in neuronal cells.

P1046/B47

Murfs selectively regulate the myosin replacement in cultured myotubes.

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In skeletal muscle cells, myofibrils play an essential role in generating contraction force by interaction with the thin and thick filaments. A single thick filament is formed by approximately 300 myosins and their associated proteins, while myosin is constantly replaced by the other in the thick filaments of myotubes/myofibers. Muscle hypertrophy is caused by the increment of the myofibril numbers in each myofiber whereas muscle atrophy leads to reducing the number of the myofibrils in myofibers. During muscle atrophy, the ubiquitin-proteasome system plays a pivotal role, in which muscle ring finger proteins (Murfs) function as E3 ubiquitin ligases responsible for identifying and targeting substrate for degradation. In the present study, we hypothesized that Murfs, which recognize Myh isoforms as substrates, altered the myosin replacement rates through the enhanced Myh degradation by Murfs. To assess the effects of Murfs on myosin replacement, expression vectors encoding cherry tagged Murf1, Murf2, or Murf3 was transfected into cultured muscle cells. We observed that exogenous expressed Murfs were incorporated into the M-lines of the thick filaments. Fluorescence recovery after a photobleaching experiment was conducted to examine the effects of Murf isoforms on the GFP-Myh3 replacement. Murf1 overexpression selectively facilitated the myosin replacement of exogenously expressed GFP-Myh3, although Murf2 or Murf3 overexpression did not. We further tested whether Murf1 overexpression altered the replacement of myosin isoforms, Cherry-Murf1 was coexpressed with GFP-Myh1, GFP-Myh4, or GFP-Myh7 in myotubes. As a result, Murf1 overexpression promoted the GFP-Myh4 myosin replacement but did not promote GFP-Myh1 or GFP-Myh7 replacement. Intriguingly, overexpression of Murf1 did not enhance protein ubiquitination. Our results demonstrated that Murf1 selectively regulated myosin replacement in a Myh isoform-dependent fashion. Our findings suggest that Murf1 might play another role in the myosin replacement in the thick filaments besides function as E3.

P1047/B48

Cargo adaptors use a handhold mechanism to engage with myosin V for organelle transport.

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The yeast myosin V motor, Myo2, is critical for organelle transport in budding yeast. Attachment and detachment of Myo2 from its cargo is regulated via organelle-specific cargo adaptors. The vacuole adaptor, Vac17, binds Myo2 and the vacuolar membrane protein, Vac8. Regulation of Vac17 plays a key role in both the assembly and disassembly of the Myo2-Vac17-Vac8 complex. Here, using genetics, cryo-electron microscopy and structure prediction, we discover that instead of solely using a single site, Vac17 binds to two sites on Myo2. Moreover, we show that the peroxisome adapter, Inp2, also requires two distinct surfaces on Myo2, and, unexpectedly, Vac17 and Inp2 overlap at one site. These data allow us to propose a "handhold" model, where the Myo2 cargoes interact with multiple sites on the Myo2 tail, which may offer tighter myosin V-cargo interactions and enable coordinated cargo binding.

P1048/B49

Myosin VI regulates IGF-1R trafficking in pancreatic cancer cells.

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Pancreatic ductal adenocarcinoma (PDAC) cells rely on signaling from overexpressed transmembrane receptors, including the insulin-like growth factor 1 receptor (IGF-1R), to promote cell proliferation and migration. GIPC interacts with the cytoplasmic tail (C-tail) of IGF-1R via a PDZ-binding motif (PBM). Disrupting the interactions between GIPC and IGF-1R has been shown to impede PDAC progression, through mechanisms that are unknown. GIPC is a multifunctional adaptor protein that engages the actin-based molecular motor myosin VI. Myosin VI is essential for timely endocytic traffic and is overexpressed in multiple cancers. Here, we dissect the regulation of myosin VI through GIPC-membrane receptor interactions and the impact of myosin VI on IGF-1R trafficking in a PDAC cell line, PANC1. We use biophysical techniques to demonstrate GIPC-receptor C-tail complexes induce significant conformational and motile changes in myosin VI by activating the motor and enhancing cargo transport. We discover the conformation of GIPC is modulated by myosin VI and IGF-1R binding. Myosin VI inhibition reduces IGF-1R internalization and trafficking to the Golgi in PANC1 cells. We find that IGF-1R kinase activity is not essential for receptor endocytosis. Our next steps are to explore strategies to target the myosin VI-GIPC interface and link myosin VI activity to cell adhesion and migration.

P1049/B50

Cerebellar degeneration-related protein 2 is a dynein adaptor that binds kinectin to regulate ER sheet organization.

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The microtubule motor cytoplasmic dynein-1 is recruited to endomembrane compartments by specific adaptor proteins, but there are currently no known dynein adaptors for the endoplasmic reticulum (ER).

We identified conserved dynein-binding motifs, including the CC1 box, at the N-terminus of cerebellar degeneration-related protein 2 (CDR2) and its paralog CDR2-like (CDR2L), onconeural antigens with poorly understood functions. Using biochemical approaches and immunofluorescence-based assays in HeLa cells, we establish CDR2/L as novel dynein adaptors and demonstrate that CDR2 is recruited to ER sheets by the integral membrane protein kinectin (KTN1). CDR2/L knockout enhances KTN1-dependent ER sheet stacking, the reversal of which by exogenous CDR2 requires its CC1 box. Exogenous CDR2 expression additionally promotes CC1 box-dependent clustering of ER sheets near centrosomes. Translation elongation factor 1-delta (EF1D) competes with CDR2 for binding to the KTN1 C-terminus, and EF1D knockdown increases endogenous CDR2 levels on ER sheets, inducing their clustering near centrosomes. These results implicate dynein in the organization of ER sheets.

P1050/B51

Mapping the intermolecular contacts in the Ndel1-Lis1-dynein complex.

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Cytoplasmic dynein-1 (dynein) is a microtubule minus-end directed motor protein that functions in mammalian cell division, intracellular cargo trafficking, and neuronal migration. While basally autoinhibited, dynein binds to the dynactin complex and an adaptor to form the "active transport complex", which is competent to walk processively on microtubules. The protein Lis1 promotes formation of the active transport complex in multiple ways, including relieving dynein's autoinhibited conformation and recruiting dynactin to dynein. Ndel1 is a dynein- and Lis1-binding protein whose effects on dynein activity have been challenging to decipher. Previous work has shown that Ndel1 negatively affects dynein activity by inhibiting dynactin-dynein binding and suggests that Ndel1 must be released before dynein is activated; in contrast, Ndel1 has also been reported to increase the dynein-activating effects of Lis1. How Ndel1 can function to both inhibit and activate dynein is an outstanding question. In this study, we sought to further clarify the mechanisms of Ndel1 and Lis1 in dynein regulation. Using a combination of quantitative pulldown assays and single molecule TIRF-based motility assays, we mapped the functional interactions of Lis1, Ndel1, and dynein. We also used cross-linking mass spectrometry and electron microscopy to determine the structure of the Ndel1-dynein and Ndel1-Lis1-dynein complexes. Our preliminary findings show that the interactions between Ndel1, Lis1, and dynein are complex, multivalent, and interdependent. The work we present here will aid in our understanding of how Ndel1 modulates dynein activity.

P1051/B52

CCSer2 Gates Dynein Activity at the Cell Periphery.

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The main minus-end directed, microtubule-associated motor in human cells is cytoplasmic dynein-1 (dynein). Because dynein is responsible for the movement of hundreds of diverse cargoes, it is essential for dynein to be able to discriminate between different cargoes and traffic them at the appropriate time and from the correct cellular space. We currently have a good understanding of how dynein differentiates between cargoes. A family of dynein regulators, called adaptors, tether dynein to unique cargoes and activate its motility. However, it is unclear how dynein activity is regulated in time or space. Here, we provide evidence that the protein CCSer2 activates dynein in a space-dependent, rather than a

cargo-dependent, manner. We show that CCSer2 promotes directional cell migration in both in-vivo zebrafish models and 2D human cell culture by facilitating the activation of cortically localized dynein. Further, through co-immunoprecipitation experiments and in-vitro binding assays using purified protein components, we establish that CCSer2 binds to and inhibits the activity of the dynein regulator, Ndel1. We hypothesize that CCSer2 at the cell periphery sequesters Ndel1 away from dynein and results in local dynein activation. Since other Ndel1 binding proteins exhibit distinct cellular localizations, we propose that CCSer2 likely belongs to a family of proteins that activate dynein across subcellular spaces through the inhibition of Ndel1.

P1052/B53

Domain specific characterization of the kinesin-2 subunit KAP3 through cell culture and immunoprecipitation studies.

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The heterotrimeric, microtubule-based molecular motor kinesin-2 is associated with a variety of systems requiring coordinated intracellular transport. Its non-motor accessory subunit, KAP3, is essential to the function of native kinesin-2, serving to link the motor subunits to specific cargoes. While well characterized in certain contexts, the details of how kinesin-2 contributes to the transport and localization of the myriad of potential cargoes present within the cell has yet to be fully elucidated. To address this, we have cloned constructs for five GFP-tagged fragments of H.s. KAP3, which collectively span the entirety of its sequence. Initial analysis of cells overexpressing these constructs revealed consistent patterns that are indicative of domain specific properties for each fragment. Current studies focus on defining how each region of KAP3 may interact with various subcellular systems using confocal fluorescence microscopy in both fixed and living samples. Our approach allows for the observation of co-localization patterns and a more detailed description of the nature and duration of these interactions. Furthermore, in vitro pulldown assays have been developed which promise to facilitate the identification of novel binding partners for KAP3. Ultimately, this work will contribute to our knowledge of kinesin-2 mediated transport with potential applications to the various roles in which it functions.

P1053/B54

Allosteric Regulation of Kinesin-1 Through D*e Novo* Protein Design.

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Kinesin-1 is a ubiquitous microtubule motor involved in intracellular cargo transport within most eukaryotic cells, with a prominent role in axonal transport. Despite continuing progress in kinesin enzyme mechanochemistry and emerging understanding of the cargo recognition machinery, it is not known how these functions are coupled and controlled by the α-helical coiled coils encoded by a large component of kinesin protein sequences. Recently, we combined computational structure prediction with single-particle negative-stain electron microscopy to reveal that the kinesin-1 heterotetramer accesses open active and closed auto-inhibited states through an equilibrium centred on a flexible elbow within the complex coiled-coil architecture. Here, we target the elbow to engineer a closed state that can be opened with a de novo designed peptide. The alternative states are modelled computationally and confirmed by biophysical measurements and electron microscopy. In cells, peptide-driven activation increases kinesin transport, demonstrating a primary role for conformational switching

in regulating motor activity. The designs are enabled by our understanding of ubiquitous coiled-coil structures, opening possibilities for controlling other protein activities. This understanding also allows us to tune the affinity of these designed interactions to control the extent of activation of engineered kinesins, opening the door to new approaches in quantitative chemical biology.

P1054/B55

Optogenetic control of kinesins and dynein reveals their specific roles in vesicular transport.

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Each cargo in a cell employs a unique set of motor proteins for its transport. It is an open question why several types of motors are often bound to the same cargo. To dissect the roles of each type of motor in intracellular transport, we developed an optogenetic system to trigger the inhibition of kinesin-1, -2, -3 or dynein with light. This system allows us to control the activity of the endogenous set of motor proteins that are bound to intracellular cargoes. We examined the effect of optogenetic inhibition of kinesins-1, -2, and -3 and dynein on the transport of Rab5-positive early endosomes, Rab7-positive late endosomes, and lysotracker-positive lysosomes. In agreement with previous studies, sustained inhibition of either kinesins or dynein results in reduced motility in both directions. However, transient, optogenetic inhibition of kinesin-1 or dynein causes both early and late endosomes to move more processively by relieving competition with opposing motors. In contrast, optogenetic inhibition of kinesin-2 reduces the motility of late endosomes and lysosomes, and inhibition of kinesin-3 reduces the motility of early endosomes and lysosomes. These results suggest that the directionality of transport is controlled through regulating kinesin-1 and dynein activity. On vesicles transported by several kinesin and dynein motors, motility can be directed by modulating the activity of a single type of motor on the cargo.

P1055/B56

The Nuclear Envelope Cargo Adapter UNC-83 Regulates the Choice Between Kinesin-1 and Dynein Driven Nuclear Movements.

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Nuclei interact with both dynein and kinesin motors, resulting in their bi-directional movement along microtubules with a net directionality. However, the mechanisms by which cargos bias their directional transport are not well understood. Nuclei move toward the plus ends of microtubules during C. elegans embryonic hypodermal development while moving toward the minus ends of microtubules in larval hypodermal P cells. UNC-83, a KASH protein found in the outer nuclear membrane acts as a cargo adaptor for microtubule motors. UNC-83 recruits kinesin-1 and dynein to nuclei in embryonic hypodermal cells and larval P cells, respectively. The process by which UNC-83 regulates these motors to produce opposite directional nuclear movements at different developmental stages remains unclear. We hypothesize that nuclear movement directionality in *C. elegans* is determined by the developmentally regulated expression of alternative isoforms of UNC-83. To test this, we used genetic and biochemical methods. Mutations affecting the long UNC-83a/b isoform disrupted dynein-dependent movements during larval P-cell nuclear migration, whereas the shorter UNC-83c isoform sufficed for kinesin-driven movement in the embryonic hypodermis. Deletion analysis revealed that residues 58-233,

specific to the long UNC-83a/b isoform, are necessary for dynein-dependent nuclear migration. Furthermore, expressing the long isoform under the control of the short isoform's promoter disrupted nuclear migration in embryonic hypodermal precursors, suggesting that the UNC-83a/b-specific N-terminal domain inhibits kinesin-1 activity. This led to the hypothesis that UNC-83a/b-specific domain of 300 residues inhibits kinesin-1 activity. We tested this model using purified components to perform microtubule gliding assays. Additionally, biolayer interferometry assays showed that UNC-83a/b has a tenfold weaker affinity for kinesin light chain (KLC-2) than UNC-83c. These findings support our model where UNC-83a/b directly inhibits kinesin-1, while UNC-83c activates kinesin-1-mediated nuclear movement. This work advances our understanding of how cargo adapters regulate the directionality of microtubule-dependent transport during development.

P1056/B57

Protein transport along microtubules by kinesin-driven shepherding.

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During long-distance transport in eukaryotic cells, cargo typically binds to motors such as kinesin for unidirectional transport along microtubules. In addition to conventional cargo, like vesicles, kinesin also shuttles non-motor microtubule-associated proteins (MAPs) to microtubule ends. Computational modeling of a system of kinesin and a MAP with no direct binding to the motor unexpectedly revealed the redistribution of the MAP to microtubule plus ends, suggesting an unconventional mode of protein transport. We recapitulated this phenomenon experimentally in a minimal *in vitro* system using a kinesin-1 (K401) and PRC1, a non-motor MAP that binds and diffuses on the microtubule but shows no detectable binding to K401. Our findings suggested that a stoichiometric excess of K401 can act as a unidirectional barrier to PRC1 diffusion. This effectively shepherds PRC1 to microtubule plus end without conventional motor-cargo interactions. Single-molecule imaging revealed unidirectional streams of PRC1 molecules over micron distances along microtubules. Remarkably, we found that shepherding occurs with low kinesin processivity, and the kinesin itself is not redistributed to microtubule ends. These findings reveal a new class of transport mechanism of microtubule-bound cargo that does not require motor-cargo binding and high motor processivity, two principles usually considered necessary for long-distance transport. Additionally, this mechanism obviates the need to recycle motor molecules from microtubule ends to the lattice, as is required for continued unidirectional cargo transport along microtubules.

P1057/B58

Phosphorylation of the dynactin DCTN5 component is required for regulation of ciliogenesis and cytokinesis via the novel dynein adaptor, Luzp1.

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Dynactin is required for binding of cytoplasmic dynein to different intracellular cargoes. Cryo-EM studies show that cargo-associated "activating adaptors" contact the dynactin DCTN5 (p25) component at distinct sites (Lau et al, 2021; Chaaban and Carter, 2022), but how adaptor binding is regulated is still poorly understood. DCTN5 knockdown and mutant rescue *in cellulo* allows us to perform a structure-function analysis of two adaptor-binding motifs: the basic loop and C-terminal alpha helix. We showed

previously that the basic loop binds adaptors for endomembrane cargoes (BICD1/2, Hook1, FIP3). The alpha helix, which is essential for viability in mice, suppresses actin dynamics and primary ciliogenesis. DCTN5 N20 is required for binding to all adaptors tested and binding between the C- and N-termini is required for interaction with some adaptors (Hook2/3, FIP3/4, spindly), consistent with previous work showing that the DCTN5 N-terminus contributes in a major way to dynein/dynactin supercomplex formation (Chaaban and Carter, 2022). Luzp1, a protein implicated in ciliogenesis, has the structural features of an adaptor (including a coiled coil and a spindly motif). BioID shows it to interact with dynein and dynactin. Supercomplex formation among Luzp1, dynein, and dynactin requires the DCTN5 N- and C-termini and the Luzp1 spindly motif. Luzp1 knockdown or rescue with point mutations in the spindly motif yields aberrant primary ciliogenesis and dysregulated actin dynamics similar to loss of the DCTN5 alpha helix. Endocytic phenonema that involve Hook1/2/3 and FIP3/4 adaptors remain unaffected. We showed previously that DCTN5/6 depletion interferes with proper positioning of anillin, septin, RhoA and citron kinase at the cleavage furrow, furrow ingression, and abscission. Luzp1 depletion or mutant rescue yielded similar phenotypes. DCTN5 S23 and S31 are phosphorylated in vivo. A S23D phospho-mimic mutant, but not phospho-null S23A supports dynein/dynactin association with Luzp1 and rescue the ciliogenesis, actin dynamics and cytokinesis phenotypes. S23 phosphorylation appears to be a regulatory mechanism selective for interactions with Luzp1, as the phospho-null S23A mutant supports dynein/dynactin supercomplex formation with all other adaptors tested and does not affect endomembrane trafficking or motility. By contrast, S31 mutagenesis reveals that phosphorylation is required for supercomplex formation promoted by BICD1/2, Hook2/3, or FIP3/4, but not Luzp1. S31 phospho-null or -mimic mutants did not affect actin dynamics. Taken together, these data extend our previous work on the novel dynein/dynactin scaffolding adaptor, Luzp1, to reveal roles for DCTN5 phosphorylation at distinct sites that contribute to regulation of actin dynamics.

P1058/B59

Lis1 relieves cytoplasmic dynein-1 autoinhibition by acting as a molecular wedge.

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Cytoplasmic dynein-1 is the major minus-end directed microtubule motor. Dynein is autoinhibited in a “Phi” conformation and undergoes a series of conformational changes to form active complexes, which consist of two dynein dimers, the dynactin complex and activating adaptor(s). The Lissencephaly 1 gene, LIS1 is genetically linked to the dynein pathway from fungi to mammals. We and others have recently shown that Lis1 is required to form active dynein complexes. Here, using cryo-electron microscopy (CryoEM), we solve a high-resolution structure of two Lis1 dimers ‘wedged’ on each side of an auto-inhibited dynein motor dimer. We named our structure "Chi" due to its resemblance to the Greek letter and because Chi, follows Phi in the Greek alphabet. Our structure reveals two new contact sites between dynein and Lis1 and is suggestive of the mechanism by which Lis1 promotes the relief of dynein autoinhibition. Using structure informed mutagenesis, we disrupt each of these sites and show that they are required for Lis1’s regulation of dynein in vivo in S. cerevisiae. To test if Chi’s function is conserved, we use in vitro reconstitution of human dynein complexes. Addition of Lis1 increases velocity and landing rates of dynein but when we disrupt the Chi interface, these increases are abolished. We propose that our structure is an intermediate state in dynein’s activation pathway. Ongoing CryoEM work reveals Chi-like structures of full-length human dynein and Lis1 proteins. One novel contact site identified between Lis1 and the dynein linker is mutated in patients with the neurodevelopmental

disease lissencephaly. Ongoing works aims to characterize how this Licencephaly mutation affects dynein function.

Protein Folding, Assembly, and Quality Control

P1059/B60

Septins from yeast to man rededicate nucleotide binding- and hydrolysis from signal transduction to protofilament integrity reporting.

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The septins are a conserved family of filament-forming guanine nucleotide binding proteins, often named the fourth component of the cytoskeleton. Human septins assemble into palindromic hexameric (SEPT2-6-7-7-6-2) or octameric (SEPT2-6-7-9-9-7-6-2) protofilaments which polymerize further into higher-ordered structures which are required for essential intracellular processes such as cytokinesis, polarity establishment, or cellular adhesion. Septins belong structurally to the P-Loop NTPases but unlike their relatives such as Ras or Rho, they do not mediate signals to effectors through GTP binding and hydrolysis. Biochemical approaches addressing how and why septins utilize nucleotides are hampered by the stability of septin complexes after recombinant expression and the lack of nucleotide-depleted complexes. Using molecular dynamics simulations, we determined inter-subunit binding free energies in human and yeast septin dimer structures and in their respective, in silico generated, apo forms. The nucleotide in the GTPase active subunits was identified as a stabilizing element as it is coordinated at its guanine ring to conserved amino acids. Removal of GDP resulted in flipping of a conserved arginine residue (termed Argβb due to its location in a septin-unique β-meander) and disruption of an extensive hydrogen bond network around the guanine ring, concomitant with decreased inter-subunit affinity. We investigated these findings experimentally in human cells. Purified SEPT7-SEPT9 and SEPT7-SEPT7 complexes bearing a Argβb(Ala) mutation did not assemble into dimers. Reintroduction of GFP-SEPT9 into a SEPT9 knockout fibroblast cell line led to incorporation of GFP-SEPT9 into the intracellular septin cytoskeleton and restored a wildtype-like phenotype. GFP-SEPT9 bearing the Argβb(Ala) mutation failed to incorporate into the septin cytoskeleton and SEPT9 knock out cells expressing this mutant remain in their knockout phenotype. To conclude, we present here a combination of in silico, in vitro and in vivo methodology to dissect how septins utilize an otherwise conserved feature (GTP hydrolysis for signal transduction) as a signal for protofilament integrity. Since this feature is conserved from yeast to man, we suggest that this rededication of nucleotide hydrolysis occurred at an early stage of septin evolution.

P1060/B61

Elucidating the Molecular Mechanisms of Septin Cytoskeleton Assembly.

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Septins are cytoskeletal guanine nucleotide binding proteins found in all eukaryotes except higher plants. Their oligomerization into apolar protofilaments is facilitated by inter-subunit contacts at two alternating distinct interfaces: the G-interface, formed between the central Ras-like guanine nucleotide binding domains (G-domains), and the NC-interface, stabilized by the N- and C-terminal extensions

flanking the G-domain. Septin protofilaments can further organize into higher-ordered structures such as gauzes or rings, which act as scaffolds or diffusion barriers in various intracellular processes, including cytokinesis, vesicular transport, and polarity establishment. Nucleotide binding and GTP hydrolysis are believed to play crucial roles in these processes, especially due to their association with various human diseases linked to mutations in the nucleotide binding pocket of septins. However, there is currently a lack of biochemical evidence elucidating the functional role of the guanine nucleotide. To address this question, we established a pipeline for the purification of isolated G-domains from all mitotic septins of the budding yeast *S. cerevisiae* and investigated their assembly and nucleotide interactions *in vitro*. All tested septin subunits were monomeric apo proteins, with two subunits exhibiting hydrolytic activity against GTP. Coexpression of compatible G-interface pairs resulted in the formation of dimeric complexes tightly associated with their nucleotides and significantly reduced catalytic activity. Removal of the bound nucleotides induced dissociation of the complexes. Consistently, G-interface formation by monomeric septins was only possible in the presence of nucleotides, showing that nucleotide binding is essential for G-interface integrity. Furthermore, we discovered that nucleotide binding and G-interface formation are also essential prerequisites for stable NC-interface formation, demonstrating direct communication between G- and NC-interfaces within the same septin subunit. Structural analysis revealed that this communication is achieved by large rearrangements in the highly conserved septin unique element (SUE). In summary, we present evidence for a septin-specific polymerization mechanism in which guanine nucleotide binding induces conformational changes in the SUE, enabling proper G- and NC-interface formation.

P1061/B62

CUL3-KLHL41 regulates the homeostasis of the sarcoplasmic reticulum in skeletal muscle.

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CUL3-RING E3 ubiquitin ligases (CRL3s) play crucial roles invarious cellular processes. The N-terminal region of CUL3 forms complexes withBric a brac, Tramtrack, and Broad-Complex (BTB)-domain proteins, whichrecognize target substrates for ubiquitylation. In the human genome, 183 genesencode BTB domain-containing proteins, with 114 having the structural basis forbinding to CUL3, and 38 known to form complexes with CUL3. This enables CRL3sto participate in diverse cellular processes. Each BTB domain protein canrecognize multiple substrates, indicating that the full scope of CRL3 functionsin cellular processes is yet to be discovered. The goal of this study is to identifyCRL3s involved in regulating endoplasmic reticulum (ER) homeostasis. A cluesupporting the role of CRL3s in the ER came from a proteomic study of skeletalmuscle-specific *Cul3 knockout* (*KO*) mice, which revealedincreased levels of SERCA1, RYR1, and other Ca^{2+}-binding proteins indiaphragm muscles. These proteins are residents of the sarcoplasmic reticulum(SR), a smooth subcompartment of the ER that plays a critical role in Ca^{2+}storage and release in skeletal muscle cells. We identified KLHL41 as apromising candidate. KLHL41 is a BTB domain protein expressed in skeletalmuscle, with a pool present at the SR in myofibers. We hypothesized that theeffect of CUL3 on the SR proteins, and thus on SR homeostasis, is mediated, atleast in part, through its association with KLHL41. When we knocked down *KLHL41*expression in C2C12 myotubes, we observed a significant increase in levels ofER-resident proteins, including PERK, IRE1, RyRs, and SERCA1. Similarly, SERCA1protein levels were elevated in zebrafish *klhl41 knockout*mutants. Additionally, the sarcoplasmic reticulum appeared enlarged in *klhl41a*;*klhl41bdouble* mutants. These findings indicate that KLHL41 plays a crucialregulatory role in maintaining SR homeostasis in skeletal muscle.

P1062/B63

Programmed nuclear pore complex replacement during oogenesis in *Drosophila*.

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All somatic cells age and eventually die. In contrast, germ cells possess the remarkable ability to reset their cellular age, thereby initiating new life. Despite this extraordinary phenomenon, the precise molecular mechanism underlying this rejuvenation process remains elusive. The germ cell-derived oocyte supplies a reservoir of "maternal components," including organelles and complex macromolecules, which play a pivotal role in initiating embryogenesis. However, it remains unclear if and how these maternal contributions are selectively composed of unaged or correct components. **Using *Drosophila* oogenesis as a model system, we found that the nuclear pore complex (NPC)—a maternally provided structure serving as a gateway between the nucleus and cytoplasm—exhibits a unique pattern.** In the female germline, NPC levels decrease before oocyte specification and subsequently increase afterward. Results from an RNAi screen indicate a crucial role for the ESCRT-III protein complex and Vps4. These proteins facilitate membrane scission and the removal of NPCs from the nuclear membrane, which are then fed into lysosomes for degradation concurrent with oocyte specification. Depletion of ESCRT-III components or Vps4 in the germline results in the accumulation of NPCs during oocyte specification, ultimately leading to the failure of oocyte specification itself. Concurrent with ESCRT-III mediated NPC degradation, there is a simultaneous rise in nascent transcription of nucleoporins that constitute the NPCs, facilitating the production of new NPCs. These findings suggest a dynamic process in which NPCs in the female germline undergo degradation and subsequent resynthesis of new NPCs. We propose that germ cells systematically turn over NPCs for their maternal contribution, thereby reversing the cellular age of one component of the cytoplasm to enable new life.

P1063/B64

Identification and functional analysis of CRED2, which leads to lysosomal degradation of aberrant extracellular proteins.

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Degradation of aberrant proteins is important for maintaining of protein homeostasis. To date, the degradation pathways of aberrant intracellular proteins are well understood from studies in the field of proteasomes and autophagy. In contrast, the protein quality control pathways that degrade aberrant extracellular proteins remain poorly understood. Only a few extracellular protein quality control factors have been reported, including Clusterin and α2-macroglobulin as extracellular chaperones. We have demonstrated the CRED (chaperone- and receptor-mediated extracellular protein degradation) pathway, in which Clusterin selectively binds to an extracellular aberrant protein and leads to lysosomal degradation via endocytosis. We hypothesized that CRED factors recognize hydrophobic regions of aberrant proteins and identified candidate extracellular chaperones by mass spectrometry analysis of plasma proteins that selectively bind to hydrophobic Sepharose. In addition, internalization assays were performed in cultured mammalian cells to monitor the lysosomal degradation of the candidate proteins with the aberrant protein complex. Among these candidates, the CRED2 protein was newly identified as a factor that recognizes, internalizes, and degrades extracellular aberrant proteins. CRED2 was involved

in the lysosomal degradation of an amyloidogenic protein, which is an intractable disease. CRED2 also had a different substrate specificity from Clusterin. Furthermore, heparan sulfate on the plasma membrane was important for the cellular uptake of the CRED2-substrate complex. Currently, we are investigating further details of the molecular and physiological functions of CRED2.

P1064/B65

PEX1^{G843D} is Sufficient to Support Peroxisome Matrix Protein Import but is Rapidly Degraded by the Ubiquitin-Proteasome System Due to an Assembly Defect With PEX6.

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The heterohexameric PEX1/PEX6 AAA-ATPase is required for biogenesis and maintenance of the peroxisome. In humans, mutations in *HsPEX1* and *HsPEX6* cause the majority of Peroxisome Biogenesis Disorders (PBDs). One missense mutant - PEX1^{G843D} - accounts for approximately 30% of observed PBDs. Although the G843D mutation is known to disrupt peroxisome matrix protein import, its effect on PEX1 function remains unknown. Here, we find that the homologous mutation in yeast, *Sc*Pex1^{G700D}, only causes a minor loss of peroxisome import function in-vivo and forms a functional ATPase capable of unfolding substrates in-vitro. We generated model cell lines possessing the PEX1^{G843D} mutation that recapitulate the phenotypes observed in patient fibroblasts and show that the G843D mutation reduces the in-vivo half-life of PEX1 due to increased proteasomal degradation. Overexpression of PEX1^{G843D} is sufficient to partially restore peroxisome import deficiency, thus indicating that PEX1^{G843D} remains functionally competent. The G843D mutation is located near the D2 ATPase domain where it may disrupt the binding of ATP and thus interaction with PEX6. Indeed, the isolated *Sc*Pex1^{G700D} D2 domain has an altered thermal-melt curve shift in-vitro that can be partially restored by high ATP concentrations. PEX1^{G843D} inefficiently assembles with PEX6, which is sufficient to trigger degradation of PEX1. Loss of PEX6 is also sufficient to trigger degradation of wild-type PEX1 which indicates a potential regulatory pathway for “orphaned” PEX1 that is exacerbated by the assembly defect of the G843D mutation. We performed co-immunoprecipitation mass-spectrometry and identified multiple E3 ligases enriched in binding to PEX1^{G843D}. A subsequent candidate CRISPRi screen showed that knockdown of these E3 ligases did not significantly abrogate degradation of PEX1^{G843D}. However, a chimera of PEX1^{G843D} and the deubiquitinase OTUB1 was sufficient to slow degradation of PEX1^{G843D}, thus hinting at a more complex regulation of dysfunctional PEX1 by the ubiquitin-proteasome network. These observations suggest that therapeutic strategies to increase PEX1^{G843D} levels, such as DUBTAC (deubiquitinase-targeting chimeras) technology, would be effective for PBDs arising from the G843D mutation.

P1065/B66

Like Mother Like Child: Passing Down Proteins And Organelles For Future Generations.

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Organelles are inherited from cell to cell to maintain organismal health. In contrast to somatic tissues, the germline poses a challenge to prevent lipid and protein organelle contents from deterioration as they are inherited between generations. Intriguingly, quality control events naturally occur in oogenesis to prevent damaged organelles from being inherited by the egg. However, eggs can be arrested for a prolonged period prior to fertilization, allowing stockpiled proteins and organelles to be susceptible to

damage before embryogenesis. Upon egg fertilization, many maternally deposited transcripts are degraded to initiate the maternal to zygotic transition in the developing embryo, while organelle protein levels remain constant throughout embryogenesis. The degree in which maternally deposited organelle proteins either persist or are recycled with zygotically translated proteins produced in the embryo remains poorly understood. Furthermore, germ cells generate zygotic transcripts much later than somatic cells, and undergo a limited number of cell divisions before progeny are mature enough to reproduce. This raises the intriguing question if inherited maternal organelle proteins are differentially regulated between the offspring's germline and soma. Through molecular and genetic analysis, I have investigated how parental organelle proteins in Drosophila are utilized and maintained in the next generation. Using acandidate-based approach, I found that germ cells receive more maternally deposited nucleoporins than somatic cells, which persist throughout embryonic and larval development. Using an unbiased pulse-chase labeling approach, I have identified a subset of maternally deposited chaperones that are long-lived in the embryo. Together, this work has revealed uncharacterized maternal proteins important for development. This research will determine the degree protein and organelle continuity is maintained between generations and aim to reveal if specialized quality control pathways exist to regulate the inheritance of maternal proteins.

P1066/B67

ATAD1 prevents clogging of TOM and damage caused by un-imported mitochondrial proteins.

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Mitochondria require the constant import of nuclear-encoded proteins for proper functioning. Impaired protein import not only depletes mitochondria of essential factors but also leads to toxic accumulation of un-imported proteins outside the organelle. Here, we investigate the consequences of impaired mitochondrial protein import in human cells. We demonstrate that un-imported proteins can clog the mitochondrial translocase of the outer membrane (TOM). ATAD1, a mitochondrial ATPase, removes clogged proteins from TOM to clear the entry gate into the mitochondria. ATAD1 interacts with both TOM and stalled proteins, and its knockout results in extensive accumulation of mitochondrial precursors as well as decreased protein import. Increased ATAD1 expression contributes to improved fitness of cells with inefficient mitochondrial protein import. Overall, we demonstrate the importance of the ATAD1 quality control pathway in surveilling protein import and its contribution to cellular health.

P1067/B68

Investigating the Role of Multimerization in Regulating Hmg2, a Key Sterol Synthesis Enzyme.

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The sterol pathway produces a variety of biomolecules that help regulate a wide range of cellular processes and disease progression. One of the key enzymes, and a popular therapeutic target, within the sterol synthesis pathways is HMG-CoA Reductase (HMGR). The yeast HMGR isozyme, Hmg2, undergoes feedback-regulated degradation as a means of controlling sterol synthesis. The Hampton lab discovered that a sterol pathway intermediate, GGPP, triggers Hmg2 degradation by inducing a reversible misfolded state that enhances Hmg2's recognition by the HRD ERAD quality control pathway. Previously, the Hampton lab showed that the quaternary structure of Hmg2 can play a role in Hmg2

regulation. Biochemical evidence revealed that a stable mutant of Hmg2 is able to suppress degradation when co-expressed with wild-type Hmg2. We aim to further understand and structurally characterize the role that quaternary structure plays in Hmg2 regulation. An AlphaFold query predicts Hmg2 to form stable homodimers along the eighth transmembrane helix and, furthermore, predicts a similar heterodimer between Hmg2 and the unregulated, stable Hmg1 isozyme. Preliminary co-Immunoprecipitation (co-IP) assays confirm that Hmg2 can indeed interact with Hmg1. The biologically relevant multimeric state of Hmg2 is unknown, but we are currently investigating multimerization with genetic and biochemical assays. For the first time we show an interaction between Hmg2 and Hmg1, but the biological role that a Hmg2-Hmg1 multimer plays is unknown. The Hampton lab has extensively revealed that Hmg1 is not responsive to GGPP or rapidly degraded by the HRD pathway like Hmg2, which led us to question if Hmg1 could suppress Hmg2 degradation when the two are co-expressed. An alternative function of Hmg2 mediating Hmg1 degradation is also being explored. We are using genetic, biochemical, and structural assays to determine the multimeric state of Hmg2, further characterize the role that multimerization plays in Hmg2 regulation, and verify the biological significance of a Hmg2-Hmg1 interaction. These studies will further our understanding of ligand-mediated degradation and reveal potential tools for pharmaceutical therapy.

P1068/B69

Defining the Scope and Mechanism of Proteostatic Control by the Fibrillar Procollagen *N*-Glycan.

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Collagen is the protein scaffold for metazoan life. Fibrillar collagens, assembled in the Endoplasmic Reticulum (ER) as procollagens, are the primary protein component of bone and skin, and their characteristic feature is an extended, non-globular triple-helical domain. The procollagen trimer is a distinctive protein complex, presenting unique folding challenges to the ER proteostasis network. While procollagen itself has sequence properties that assist in guiding its assembly, the process is challenging and error-prone, with procollagen-expressing cells estimated to degrade 10-40% of newly synthesized procollagen. As such, extensive engagement with the ER proteostasis network is necessary for procollagen folding, secretion, and degradation, particularly in environments where procollagen assembly faces additional proteostatic challenges, such as development and disease.
One key feature necessary for collagen secretion under challenging folding conditions is an evolutionarily conserved *N*-glycan. Here, we investigate the extent to which and the molecular mechanism(s) by which the *N*-glycan and associated *N*-glycan chaperones (termed the ER lectin chaperones) modulate procollagen proteostasis. In preliminary experiments, where we express triple-helical domain (THD) disease variants of type-I procollagen in HT-1080 fibrosarcoma cells, we find that misfolding-prone THD variants of procollagen rely on the evolutionarily conserved *N*-glycan for secretion. We have also made progress towards understanding the role of the ER lectin chaperone network in collagen proteostasis by generating collagen-producing CRISPR interference (CRISPRi) lines, where we can selectively knock down expression of lectin machinery and examine effects on collagen assembly and secretion. We are currently working to expand upon our initial findings by deconvoluting the role(s) of lectin machinery in promoting folding *versus* quality control of misfolding-prone procollagen.

P1069/B70

Nopp140: Bridging RNA Pol I Transcription and pre-rRNA Processing.

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The nucleolar phosphoprotein of 140 kDa (Nopp140) is a ribosome assembly factor; its depletion causes nucleolar stress due to a failure in ribosome biogenesis that then disrupts homeostasis in cells with high ribosome demands such as embryonic neural crest cells or adult bone marrow stem cells. Their loss leads to disease-states called ribosomopathies. The function of Nopp140 is largely ascertained from its colocalizations and interactions with other nuclear and nucleolar components. One study suggests that Nopp140 serves as a nucleolar scaffold for recruiting RNA Polymerase I (Pol I) machinery to arrange active rDNA gene clusters. However, questions on how this mechanism occurs and what other nucleolar factors are involved remain unanswered. Nopp140 has a large intrinsically disordered central domain consisting of heavily phosphorylated, low amino acid complexity motifs. Similar motifs constitute the extended carboxyl terminal domain (CTD) of RNA Pol II. We speculate that Nopp140 may be a molecular link between Pol I transcription and pre-rRNA processing by acting in a manner like the CTD of RNA Pol II. Two isoforms of Nopp140 exist in *Drosophila melanogaster*: Nopp140-RGG and Nopp140-True, both play a role in ribosome assembly. Here, we used the entire rDNA intergenic spacer (IGS) sequence and core promoter fused either to the rDNA external transcribed spacer (ETS) followed by a portion of the 18S region and then the bacterial CAT cDNA or to just the CAT cDNA. These two constructs were separately inserted into the distal regions of *Drosophila's* 3rd chromosome. Our goal was to determine if these transgenes can form ectopic nucleoli capable of producing ETS-18S-CAT RNA or just the CAT RNA alone. Fluorescence *in situ* hybridization (FISH) with ETS and CAT probes revealed that both transgenes generate ectopic nucleoli in polypoid cells. Additionally, RpL135, a large Pol I subunit, and both Nopp140 isoforms located to these ectopic nucleoli. Fibrillarin, a pre-rRNA processing component, co-localized with ectopic nucleoli expressing the ETS-18S-CAT RNA, but not with those nucleoli expressing only the CAT RNA. These findings suggest that both Nopp140-True and Nopp140-RGG associate with the Pol I transcription machinery regardless of the RNA transcribed. We are now using a *Nopp140* gene deletion, a CRISPR-disrupted *Nopp140* gene and the ectopic nucleoli to further determine Nopp140's role in linking pre-rRNA transcription and processing *in vivo*.

P1070/B71

Mapping the Transcriptional Effects of the ER Stress Sensor IRE1 With Optogenetic Oligomerization and Long-Read Sequencing.

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The Unfolded Protein Response (UPR) is a eukaryotic stress response pathway that can switch between promoting cellular homeostasis and regulated cell death in response to imbalances in the endoplasmic reticulum (ER). Inositol-requiring enzyme 1 (IRE1) is one of three known ER membrane-resident stress sensors, and its activity in response to stress is thought to promote cell resilience and adaptation by promoting the cytoprotective aspects of the UPR. IRE1 responds to unfolded proteins in the ER lumen by forming homo-oligomers that enable trans-autophosphorylation and activation of the cytosolic RNase domain, which then splices the mRNA of transcription factor XBP1 into an active isoform with cell-wide effects. IRE1 has been shown to play an important role in diseases where cells are faced with chronic ER stress, such as neurodegeneration and diabetes. Moreover, IRE1 hyperactivation in several types of

cancer promotes the survival of tumor cells by blocking the UPR from triggering programmed cell death. A lack of direct experimental means of activation has made it difficult to study IRE1 signaling independently of the other UPR sensors, leading us to engineer an IRE1 construct with an added light-inducible oligomerizing domain to cluster and subsequently activate the cytosolic RNase domain. We used long-read sequencing to analyze the transcriptome of human cells expressing this IRE1 construct and subjected to varying exposures of light or chemical stress. We discovered potential new players in the IRE1 signaling pathway and our data support the hypothesis that IRE1's RNase domain can degrade a broad set of mRNAs in addition to *XBP1*, its canonical target. Additionally, this method opens the possibility for direct analysis of alternative splicing patterns and identification of potential new mRNA substrates of IRE1. By using a specific and direct method to investigate the role of IRE1 signaling in the complex network of the UPR, our data provide insights into the role of IRE1 in the human cell's ability to adapt and respond to severe ER stress and may guide the development of future therapeutics.

P1071/B72

Role of p53 oligomerization in its cellular localization in the presence of AML-associated NPM mutant.

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Tumor suppressor p53 plays a primary role in the cell response to DNA damage. Mutation of the TP53 gene is very frequent and leads to p53 inactivation, which is a key step in over half of human cancers. p53 is present as a monomer, dimer and tetramer in cells. Its defective oligomerization influences cell decision between proliferation, growth arrest and apoptosis. While monomeric mutants exhibit high cancer-inducing potential, consequences of a partially impaired oligomerization are mostly unexplored. Correct p53 function is related to its localization. Active p53 is stabilized in the nucleus, its degradation occurs in the cytoplasm. The localization is controlled by multiple factors including co-transport mediated by interaction partners.
Acute myeloid leukemia (AML)-related mutation of NPM (NPMmut) results in mislocalization of both NPMmut and p53 proteins to the cytoplasm, which could be one of leukemia initiating factors. Since both proteins execute their functions as oligomers and p53 abnormalities are rather frequent in AML, we investigated the role of perturbed oligomerization in the p53 mislocalization process by live-cell fluorescence lifetime imaging (FLIM), fluorescence anisotropy imaging (FAIM) and fluorescence cross-correlation spectroscopy (FCCS), complemented by immunochemical methods.
Using a set of fluorescent p53 variants with different oligomerization status, silenced NES or NLS signaling, we documented that monomeric p53 has always nuclear localization independently of the presence of NPMmut or the NES integrity. Also the p53 dimer is not mislocalized by NPMmut. The cytoplasmic translocation was observed only for oligomeric p53 variants exhibiting high oligomer stability and stronger interaction with NPMmut. We propose that p53 oligomer interacts with NPMmut monomer since the translocation process is independent of the oligomerization status of NPMmut.
(The work was supported by the Czech Science Foundation, grant 22-03875S).

P1072/B73

***In vitro* calcium requirements for actuation of ultrafast contractile protist myomenes using a novel photo-stimulation microscopy assay.**

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Many unicellular protozoans perform ultrafast motility and contractions using novel calcium-responsive filament systems termed myonemes. These systems harness calcium-binding to EF-hand proteins to drive some of the fastest known cellular motion in cell biology, on the order of cm/s velocities, yet little is known about the mechanism and regulation underlying the assembly and function of myonemes owing to the challenges of reconstituting their responsiveness *in vitro*. To overcome these challenges, we developed a microscopy-based in vitro assay that uses a photoactivatable caged Ca^{2+} chelator to locally generate spatiotemporal calcium signals upon patterned UV illumination. We used this assay to trigger formation of and quantitatively investigate calcium-dependent network formation of a model EF-hand/centrin type protein from Tetrahymena, Tcb2. By quantitatively calibrating the calcium released in this assay using the fluorescent indicator dye Rhod-5N, we determine a critical concentration of calcium-bound Tcb2 necessary to actuate network formation *in vitro*. Applying this approach to a panel of mutants that disrupt different calcium binding sites within Tcb2, we find that binding sites within the C-terminus are critical for assembly, while N-terminal binding sites appear largely dispensable. This suggests different functional roles (contraction and regulation) within the organization of the calcium-binding sites in Tcb2, like other canonical EF-hand proteins. Our approach provides new quantitative insights into the biochemical mechanism and regulation of novel myoneme contractile systems and suggests general principles for how regulated calcium-binding can be used to perform work in both natural and engineered biochemical and cellular systems.

Cell Adhesion and Migration 1

P1073/B75

Actin filament length is crucial in mesenchymal but not in amoeboid migration.

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The ability of cells to move is critical for a wide variety of cellular tasks including the search of immune cells for pathogens and the reorganization of cells in tissue development. The cytoskeletal protein actin is important for cellular migration as it is involved in its underlying mechanics. Alterations of the actin network therefore might have an impact on the migratory behavior of cells. Here, I present the effects of the stabilization and elongation of actin filaments on migrating RPE-1 cells using miuraenamide A. I will show that mesenchymal migrating cells move at lower speed with less persistence, while amoeboid migrating cells do not change their behavior. After treatment with miuraenamide A, we observed elongation of actin fibers in RPE-1 cells on micropatterns. We then performed fluorescence recovery after photobleaching measurements and saw that cells with stabilized actin filaments show a reduced

plateau value and an increased half recovery time. Thus, we conclude that miuraenamide A leads to a reduction in actin dynamics in the cell [1]. Cells with longer and more stable actin filaments have more but smaller focal adhesions. To test the effect on adhesion properties, we performed single-cell force spectroscopy. Cells with smaller focal adhesions showed lower adhesion strength and energy, suggesting that actin filament length is important for adhesion-based migration but negligible for friction-based migration. Additionally, we placed RPE-1 cells on micropatterns on a soft polyacrylamide gel and measured their contractility. We did this by comparing the initial area of the micropatterns with the area of the pattern after the cell deformed it. RPE-1 cells treated with miuraenamide A did not show any changes in contractility, from which we conclude that stabilizing actin filaments does not interfere with cellular contractility. This work emphasizes the different role of actin in mesenchymal versus amoeboid migration and adhesion and might help to influence all processes involving migration. [1] Baltes C, Thalla DG, Kazmaier U and Lautenschläger F (2022) Actin stabilization in cell migration. *Front. Cell Dev. Biol.* 10:931880. doi: 10.3389/fcell.2022.931880

P1074/B76

From Mesenchymal to Amoeboid: The Role of Actin Reorganization.

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Cell migration is a fundamental process involved in various physiological and pathological events, including embryonic development, immune surveillance, and tumor progression. During tumor progression, epithelial cells undergo epithelial-to-mesenchymal transition (EMT) to become motile. Some mesenchymal cancer cells further acquire amoeboid characteristics, such as high invasion and proliferative capacity to adopt cancer stem cell-like properties and drug resistance through a process termed mesenchymal-to-amoeboid transition (MAT). Amoeboid cancer cells exhibit high deformability and several hallmarks of cancer stem cells, enabling them to invade challenging environments and resist physicochemical stresses. However, the detailed mechanism of MAT remains elusive. In this study, we revealed that TKS5, a critical promoting factor for the invasion and metastasis of cancer cells in vitro and in vivo, plays distinct roles in mesenchymal and amoeboid migration. TKS5 functions as a scaffold protein, linking extracellular signaling through phosphoinositide binding to intracellular actin polymerization machineries via its multiple SH3 domains and Src-mediated phosphorylation, thereby coordinating actin cytoskeleton reorganization and external signals. Using a 3D microchannel system to study MAT, we discovered that TKS5 is essential for the amoeboid migration of cancer cells, particularly in the transition from the mesenchymal to the amoeboid state. Through TKS5 interactome profiling, we identified potential TKS5 effectors that regulate actin cortex deformation, which is critical for MAT. Our findings demonstrate that TKS5 plays dual roles in cancer cell migration: facilitating invadosome formation for mesenchymal migration and regulating the actin cortex for amoeboid migration.

P1075/B77

Actin viscoelasticity governs myosin-independent ECM stiffness sensing.

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Several studies have indicated a heightened response to increased substrate stiffness, which has been explained through a "molecular clutch" model. This model attributes myosin II contractility as the primary factor driving retrograde flow and accounting for stiffness-dependent differential traction.

However, experimental data demonstrate that even in the absence of myosin activity, cells are capable of transmitting a small but significant traction force, which increases with greater substrate stiffness. Actin assembly at the barbed end of F-actin can also contribute to retrograde flow by pushing against and being pushed by the membrane. A new molecular-clutch model was developed to predict this behavior, considering the role of polymerizing actin and its viscoelasticity in myosin-independent stiffness-dependent force transmission.
Experiments using traction force microscopy (TFM) revealed that WT NIH 3T3 fibroblasts and cells treated with blebbistatin exhibited increased traction with greater substrate stiffness. The myosin-independent, stiffness-dependent traction was reduced with inhibition of either Arp2/3 or formin, in addition to myosin activity. Imaging F-actin deformation through quantitative fluorescence speckle microscopy (qFSM) showed that retrograde F-actin flow speed was regulated by ECM stiffness in WT cells and those with myosin or/and Arp2/3 inhibition, with the stiffness-dependent flow difference abolished when formin activity was inhibited in addition to myosin inhibition.
Experiments found that Arp2/3 inhibition, in addition to myosin, reduced traction magnitude by approximately 4-fold compared to cells with myosin-only inhibition while minimally reducing F-actin flow. Additionally, a novel molecular clutch model was developed, where F-actin's viscoelasticity contributes to the transfer of actin motion to traction force. These findings suggest that stiffness-dependent differential tension could be induced early in nucleated NAs independently of myosin and other mechano-signaling outputs.

P1076/B78

Macrophage invasion into tissue organoids is enabled by cell divisions.

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Tissue-resident macrophages arise early in development and infiltrate all tissues in the body. Their dissemination relies on the ability to invade into interfaces between juxtaposed tissues and to migrate within densely packed environments without preexisting paths [1,2]. How tissue macrophages achieve this dissemination has been unclear. By studying invasive migration of macrophages in *Drosophila* embryos, we showed recently that the macrophage can penetrate a sealed tissue barrier only at sites of cell division. We revealed that the key mechanism by which division opens the door for macrophage infiltration is the disassembly during mitosis of the focal adhesions that attach a cell to its neighbors [3]. To investigate if this mechanism functions in macrophage infiltration in other systems, we studied co-cultures of murine macrophages with various tissue organoids. We found that macrophages invaded organoids of murine lacrimal gland, salivary gland, pulmonary airway and pancreatic tumor. Cellular resolution imaging revealed that invading macrophages were flanked by dividing cells. Macrophage invasion was preceded by a division-specific increases of the intercellular spaces and reorganization of the cell's actin cytoskeleton and cell-cell junctions. Moreover, more macrophages were found inside the faster dividing organoids compared to slower dividing organoids. And pharmacological inhibition of the cell cycle in organoids reduced the number of invading macrophages.Taken together, our findings in tissue organoids support the model that cell division is a key regulator of macrophage tissue invasion, and suggest that regulation of macrophage invasion by cell division is a broadly applicable principle that may act during development, inflammation, and tumor growth.
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P1077/B79

The Role of NDR1/2 in Regulating Cell Morphology and Polarity.

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Precise control of cell morphogenesis and polarized cell growth is crucial for development and tissue architecture. Conserved NDR (nuclear dbf2-related) kinase pathways are important for regulating cell morphology and polarity across eukaryotes. Disruptions in cell morphogenesis and polarity serve as hallmarks of many human diseases. Here, we report that human NDR1/2 kinases control cell morphology and polarity in human fibroblasts. We found that decreasing NDR1 or NDR2 protein levels changes cell size, shape and the actin-cytoskeleton in human primary fibroblasts. To further understand the role of NDR kinase on cell morphology, we assessed cell behavior on substrates of varying stiffness, and we observed that the loss of NDR1/2 results in a reduction in cell size on both 1 kPa and 40 kPa hydrogels. This effect is particularly pronounced on low-stiffness substrates, where cell spreading ability is significantly disrupted. Furthermore, we found that NDR kinase knockdown disrupts cell migration persistence and migration speed in live cell recordings.Consistent with these changes in cell morphology, we found that knockdown of NDR1 or NDR2 changes Rho GTPases activity, in particular increasing the activity of Cdc42 and Rac while decreasing RhoA activity. Further, to study Cdc42, sparse seeded cells that express a Cdc42 biosensor were time-series recorded and analyzed. In this experiment, we found that NDR1/2 kinases can temporally and spatially control the dynamic activity of Cdc42 GTPase. **In conclusion**, our preliminary data suggest that decreasing the expression levels of NDR kinases in human fibroblasts alters cell morphogenesis and cell polarity by regulating the activity of Rho-family GTPases, in particular Cdc42 GTPase. Since our lab previously reported that the fission yeast NDR kinase Orb6 controls Cdc42 GTPase, our findings in human fibroblasts highlight the conservation of these control mechanisms across evolution, from yeast to human cells.

P1078/B80

Regulation of Rap1 GTPase signaling during collective epithelial migration.

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Collective cell migration is critical for embryonic development and tissue repair. However, cells can also use the same migration machinery for spreading cancer, resulting in metastasis. To understand the molecular mechanisms that drive collective cell migration, we investigate wound healing in epithelial tissue using the Drosophila embryonic epidermis. Wounds are generated by ablating 4-5 epidermal cells in stage 14 embryos using a high powered laser. The lesions heal rapidly without scarring or inflammation, forming a supracellular actomyosin cable connected by reinforced adherens junctions at the leading edge of the migrating cells. Our previous work has demonstrated a role for the small GTPase Rap1 in coordinating the cell adhesion and cytoskeletal rearrangements required for rapid wound healing. Rap1 GTPase is misregulated in many invasive cancers, and the Rap1 signaling pathway plays a role in epithelial integrity. Thus, understanding the factors that promote Rap1 activity is critical for important questions in human health and disease. Rap1 is known to be regulated by specific GEFs - PDZ-GEF, Epac, and C3G - and a GAP - Rapgap1. RNAi-mediated knockdown of PDZ-GEF had no effects on the

rate of embryonic wound closure, cytoskeletal polarization, or cell adhesion remodeling. Visualizing PDZ-GEF using an endogenous GFP tag shows no localization to the wound edge. Together this suggests that other GEFs and GAPs are playing a more important role in activating Rap1 to drive migration. Future work will examine localization and RNAi-mediated knockdown of Epac, C3G, and Rapgap1 to better understand the regulation of Rap1 during collective cell migration.

P1079/B81

Mechanistic Study of ER-PM Contact in Cell Migration.

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Directional cell migration is crucial for processes such as development, immune responses, and cancer metastasis. Cells respond to environmental cues by polarizing, forming membrane protrusions, and migrating. Receptor tyrosine kinases (RTKs) trigger this process by activating PI(3,4,5)P3 signaling and stimulating small GTPases like Cdc42 and Rac at the leading edge, leading to actin polymerization and directed migration. Simultaneously, signaling at the cell rear must be inhibited to maintain proper directionality, but the mechanism for this inhibition is not fully understood. Recent work from Dr. Gong in our lab suggests that ER-PM contact site gradients, termed MAPPER gradients, play a key role in suppressing RTK signaling at the rear by facilitating the interaction of ER-localized PTP1B with the cell membrane, thus dephosphorylating pRTK and directing migration. We hypothesize that ER-PM contact density regulates RTK signaling and cell migration, with lowering of the contact density being critical for initiating protrusions at the cell front. To test this, I employed circular micro-patterns to guide 2D migration of single RPE-1 cells, ensuring consistent directional movement. Live-cell imaging revealed a strong correlation between cells turning the front during migration and increased PIP3 levels, with MAPPER density inversely correlated with increased membrane protrusion. Analysis showed that a 20% reduction in MAPPER at the front was necessary to initiate directional protrusion, with MAPPER punctate disassembly occurring 20 minutes before the protrusion, while peak PIP3 signals lagged by 15 minutes after maximal velocity was reached. Further, knocking down ER-PM contact forming E-syt1 in RPE-1 cells resulted in both a reduction in MAPPER density and migration velocity, underscoring the importance of ER-PM contact regulation. These knockdown cells exhibited inconsistent movement and lacked the correlation between MAPPER and migration velocity seen in control cells, confirming the critical role of MAPPER in maintaining directional migration. I investigated the relationship between EGFR phosphorylation and MAPPER dynamics by measuring the local phosphorylation at the EGFR sites pY1068, pY1148, pY1173, and pY992. Using immunofluorescence and imaged under TIRF microscopy, I found that particularly pY1068 was sensitive to EGF stimulation and the degree of phosphorylation was greatly enhanced in the region of local membrane protrusion, demonstrating a critical role between local EGFR activation, and lowering of the ER-PM density. Building on these results, my future work will focus on determining whether targeted changes of ER-PM contact density, local EGFR signaling, and membrane protrusion orchestrate the turning of the front of migrating cells.

P1080/B82

Small GTPases Arl4A/D modulate phosphoinositide conversion at the plasma membrane via INPP5E.

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Arf-like 4 (Arl4) are small GTPases known for their role in vesicle transport, actin remodeling, organelle integrity, and cell migration. INPP5E acts upstream of inositol phosphate dephosphorylation and plays an essential role in controlling ciliary growth and phosphoinositide 3-kinase (PI3K) signaling. In this study, we show that Arl4A/D interacts with INPP5E to mediate membrane recruitment of PI(3,4)P2-dependent Tsk5. We first show that INPP5E is a novel effector of Arl4A/D and is recruited to the plasma membrane by Arl4A/D. We show that Arl4A/D promotes PI(3,4)P2 production via INPP5E. The scaffolding protein Tks5 interacts directly with PI(3,4)P2 to recruit other actin polymerization proteins and promote invadopodia formation. Finally, we show that Arl4A/D promotes the translocation of Tks5 to the plasma membrane via INPP5E. Our study shows how Arl4A/D and its effector INPP5E coordinately modulate phosphoinositide remodeling and recruit Tsk5 to the membrane for the regulation of cell motility.

P1081/B83

Mechanisms of Rac-mediated motility and cellular cannibalism *in vivo*.

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The small GTPase Rac is hyperactivated in many types of cancer, and an activating mutation in Rac1 is the third most common driver mutation in sun-exposed melanoma. Metastatic melanoma cells are migratory and cannibalistic. Rac is critical for both engulfment and migration, yet how cells modulate Rac activity to elicit each behavior *in vivo* is unclear. Here, we investigate interactions between migratory border cells with germline cells, called nurse cells, in the Drosophila egg chamber to elucidate mechanisms that determine migration versus engulfment. Border cells migrate collectively in tiny spaces between the nurse cells to reach the oocyte. The nucleus, the stiffest organelle, impairs migration in confined spaces *in vitro*; how border cells move nuclei between nurse cells was not known. We hypothesized that Rac-mediated protrusions help border cells pry open spaces between nurse cells. Rac is activated locally in the leading cell to generate F-actin rich protrusions. Live imaging revealed that leading protrusions expand spaces between nurse cells prior to nuclear movement. Then, the leading cell's nucleus transiently deforms as it moves into the protrusion while nuclei of following cells and cells expressing a dominant negative Rac deform less. Surprisingly, depletion but not overexpression of lamins, intermediate filaments that support nuclear stiffness, impedes border cell migration and stabilization of leading cell protrusions. Cortical myosin II, an actin motor, accumulates behind the nucleus to move it into the protrusion. These data support a model where nuclear movement stabilizes Rac-dependent protrusions, helping to wedge open spaces between nurse cells. While Rac is required for protrusions and border cell migration, expression of constitutively active Rac (CA-Rac) prevents migration and rather induces engulfment and death of the entire egg chamber. CA-Rac border cells cannibalize small, neighboring cells and kill the nurse cell syncytium. How CA-Rac border cells kill the entire germline was not clear. Here, we report that as they move, wildtype border cells engulf small pieces of nurse cells whereas CA-Rac border cells take larger bites. CA-Rac border cells induce nuclear fragmentation in nurse cells that initiates at border cell/nurse cell contacts and spreads rapidly through the syncytium. We are currently testing the hypothesis that the activating Rac1 P29S mutation in metastatic melanoma contributes to the known cannibalistic behavior of these cells. Together, this research suggests that differential Rac activity determines whether cells migrate, engulf small fragments, or eat whole cells and may shed light on mechanisms driving cancer cell cannibalism.

P1082/B84

Spatio-Temporal Coordination of Rho GTPase Activity in Cell Migration.

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Dynamic cell shape changes play a central role in many physiological processes, such as cell migration. Small Rho GTPases are thought to be master regulators of such shape changes. In particular, the GTPase Rac1 stimulates cell protrusions, while the GTPase RhoA stimulates cell retraction. Earlier studies suggested that Rac1 and RhoA mutually inhibit each other, which was thought to segregate protrusion and retraction in distinct cell areas, leading to a stable cell polarization during directional migration. However, more recent, optogenetics-based investigations from our lab surprisingly revealed that Rac can activate Rho, which is incompatible with the idea of mutual inhibition between these molecules. Conceptually, this Rac1-dependent activation of RhoA is not suitable to stabilize cell polarization, but instead can stimulate highly dynamic cycles of protrusion and retraction, which are typical of cells migrating via a less directional and more exploratory mesenchymal mode. Here, we present a more detailed investigation into the underlying mechanism of Rac1-activity dependent Rho activation. We show that the RhoA activating GEFs, Arhgef11 and Arhgef12 are recruited to the plasma membrane by active Rac1 and thereby could play a role in mediating the observed activity crosstalk between Rac1 and RhoA. Mutagenesis studies further revealed that the plasma membrane recruitment of Arhgef12 is mediated by its PH domain, which was previously suggested to directly bind to active Rho GTPases. In contrast, Arhgef11 is recruited to sites close to the plasma membrane downstream of Rac1-induced actin polymerization via its unique F-actin binding (FAB) motif. Increasing the expression level of these GEFs stimulated protrusion-retraction dynamics and siRNA-mediated depletion inhibited protrusion-retraction dynamics, which was accompanied by an increase in migration directionality. Our study therefore shows that Arhgef11 and Arhgef12 facilitate a less directional and more exploratory mode of cell migration. These regulators mediate this effect by coordinating the central cell morphogenic processes of cell protrusion and retraction by coupling the activity of the associated small GTPases Rac1 and RhoA. The loss of directionality that is induced by the cancer-associated regulators Arhgef11/12 could thus play a role in the misregulation of cell migration during metastasis.

P1083/B85

Role of TXBP-3 in regulating spermathecal contractility through Rho Signaling in C. elegans.

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TXBP-3/TAX1BP3 is a small, highly conserved protein consisting of a single PDZ domain, which may interfere with interactions between PDZ-containing proteins. The human ortholog participates in various signaling pathways, impacting adhesion, migration, polarity, and cell proliferation. In C. elegans, TXBP-3 is expressed in multiple contractile tissues, including the pharynx, spermatheca, spermatheca-uterine valve, and rectal epithelium. We analyzed the role of TXBP-3 in the spermatheca, which is the site of fertilization in the hermaphrodite reproductive system. When txbp-3 is deleted, the transit of oocytes through the spermatheca is delayed. Conversely, overexpression of TXBP-3::GFP leads to rapid transit of

oocytes. Analysis with a genetically encoded Rho sensor suggests that TXBP-3 inhibits Rho activity. RNAi depletion of Rho kinase/LET-502 leads to low contractility in the spermatheca and retention of embryos. However, with the loss of let-502 and txbp-3, fewer embryos are retained, suggesting relatively stronger spermathecal contractility. These results suggest that TXBP-3 regulates spermathecal contractility through Rho.

P1084/B86

Deciphering the Unique Role of Src Family Kinases in the Regulation of Cell Polarity and Migration.

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An essential function of cells is to acquire polarity during cell migration, a critical process implicated in many biological processes, from development to wound healing, and is often mis regulated in human diseases such as cancer. Migrating cells exhibit front-rear polarity through the organization of cytoskeleton components and formation of adhesion structures. Important mediators in regulating polarity are tyrosine kinases. The family of Src non-receptor tyrosine kinases fine tunes signaling pathways to help execute adjustments needed for the cell to adapt to changing environments which can lead to proliferation, differentiation, apoptosis, or migration. The Src family kinases (SFKs) make up 9 members in vertebrates with Src, Fyn, Lyn, and Yes being ubiquitously expressed while the others are restricted to hemopoietic origin. Deletion of individual SFKs shows minor changes in cell function, while a deletion of *Src*, *Fyn*, and *Yes* genes together cause embryonic lethality indicating functional redundancy. While the SFKs can compensate for one another, they are expressed to different degrees across cell types and shown to have distinct functional capacities. The SFKs have very similar structures and regulatory domains but can differ in their subcellular localization. Their functional redundancy makes it challenging to decipher the specific role of each individual SFK. To address this limitation, our lab has recently built and published a light regulated (LightR) construct of Src termed LightR Src. This tool allows high temporal and spatial control of Src's enzymatic activity through blue light. Application of this tool uncovered Src's ability to induce cell polarization and directed migration. By applying this technique to Fyn, Lyn, and Yes, we are defining specific roles of individual SFKs in the regulation of cell polarization and migration. My goal is to define how local activation of Src, Fyn, Lyn, and Yes influences morphological processes mediating cell migration. This will allow us to identify unique signaling pathways the SFKs regulate at a subcellular level.

P1085/B87

Investigating Roles of Cytoskeletal Genes in *C. elegans* Sex Myoblast Development.

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The cytoskeleton is crucial for mechanical roles in cell migration, division, and morphogenesis throughout animal development. However, our comprehension of how cytoskeletal behaviors are regulated *in vivo* is still limited in comparison to our knowledge of mechanisms *in cellulo*. Here, we aim to characterize developmental roles of candidate genes associated with the cytoskeleton using an *in vivo* model system. To achieve this, we investigated the morphogenesis and differentiation of the sex myoblast (SM) cells in *C. elegans.* The SM cells originate from the M mesoblast cell and are born in the posterior region of the L1 larva. SMs then migrate anteriorly until they reach the gonad at the center of

the worm. Once there, the SM cells re-enter the cell cycle, divide three times and differentiate into the uterine and vulval muscle cells, which are essential for egg laying. Perturbations affecting SM cell migration, division, and/or differentiation can result in decreased egg counts, which can be quantified at the plate level. Cytoskeletal regulatory proteins are likely crucial for various aspects of SM development including migration, division, and regulation of membrane dynamics. To investigate the roles of candidate cytoskeletal regulators in SM differentiation, we are performing a targeted RNAi screen of approximately 120 genes associated with the cytoskeleton. For this purpose, *C. elegans* were grown on RNAi plates targeting a specific cytoskeletal gene, and scored as young adults for phenotypes including sterility, larval growth slow, protruded vulva, uncoordinated, lethal, dumpy, and bag of worms. We used brood size as a proxy for egg-laying ability based on the hypothesis that decreased brood sizes likely indicate defects in SM development. Preliminary data show depletion of key actin regulators including *cdc-42, ced-10 (Rac), unc-73 (RhoGEF-kinase), wve-1 (WAVE), Arp2/3* complex components, *unc-60 (Cofilin), ani-2, ani-3 (Anillin)*, and *nmy-2* impact development and brood size. Septin genes, *unc-59 (septin-7)* and *unc-61 (septin-8)* and genes involved in nuclear positioning *sun-1 (SUN5)* and *amph-1 (BIN1)* also impact development and brood size. These data suggest that key actin regulators, septins and proper nuclear positioning may be required for proper SM cell differentiation. Future experiments will investigate the types of defects underlying these phenotypes through further RNAi experiments and imaging. In conclusion, we can take advantage of the unique attributes of the SM cell lineage to dissect how the cytoskeleton is required for development *in vivo*.

P1086/B88

Role of actin cytoskeleton on formation of branches and PAPs of astrocytes.

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Astrocytes are the predominant type of glia in the central nervous system and have long branched stem processes (branches emerging from soma and branchlets as secondary structures generating from branches) and peripheral astrocyte processes (PAPs) with actin-rich cytoskeletal structures in vivo. PAPs contact neurons and other glial cells and have various functions like regulation of neuronal excitability. The detailed mechanisms underlying these cell-cell interactions via formation of branches and PAPs are not clear. This may be due to the complex 3D structures of astrocytes in vivo, as well as the morphology of a common astrocyte culture method (in conventional DMEM supplemented with FBS) generating fibroblast-like cells without stem processes and PAPs. Previously, we reported that chicken astrocytes cultured in Neurobasal medium with b-FGF and B27 formed branches, branchlets, and actin-rich structures similar to PAPs (Tsukuda et. al., 2019). In the current study, we examined various transfection methods (electroporation, lipofection, and lentiviral infection) maintaining stem processes and PAPs at a low cell density. Electroporation was easy and effective for analyzing localization of multiple proteins. In contrast, lentivirus infection adequately retained cell morphology although infection rate was low. Transfection and immunostaining showed that ezrin (a PAP marker), lasp-2 (a putative ezrin-interacting protein), and actin were concentrated in the process tips. Ezrin was also localized throughout the membrane in microvillus protrusions in the cell periphery. Additionally, ezrin and lasp-2 localized in different regions of actin filament in the tips, suggesting both may coordinate in elongation of stem processes. We also analyzed whether branch formation depends on integrin adhesion complexes (IACs) using astrocytes cultured on poly-L-lysine (PLL) or PLL plus laminin (PLL-L) substrates. We observed that

laminin promoted branch elongation in early stages of culture. Moreover, in process tips of astrocytes on PLL, paxillin was rarely observed, whereas on PLL-L, paxillin was colocalized with lasp-2. These results suggest the possibility that astrocytes extend branches via interaction between IACs and extracellular matrices in vivo. Conversely, in both astrocytes on PLL and on PLL-L, paxillin did not localize in collateral protrusions, suggesting that generation of branchlets is independent of common IACs.

Membrane Trafficking and Vesicular Transport 1

P1087/B89

CCDC32 stabilizes clathrin-coated pits and drives their invagination.

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Clathrin-mediated endocytosis (CME) is essential for maintaining cellular homeostasis. Previous studies have reported more than 50 CME accessory proteins; however, the mechanism driving the invagination of clathrin-coated pits (CCPs) remains elusive. Quantitative live cell imaging reveals that CCDC32, a poorly characterized endocytic accessory protein, regulates CCP stabilization and is required for efficient CCP invagination. CCDC32 interacts with the α-appendage domain (AD) of AP2 via its coiled-coil domain to exert this function. Furthermore, we showed that the clinically observed nonsense mutations in CCDC32, which result in the development of cardio-facio-neuro-developmental syndrome (CFNDS), inhibit CME by abolishing CCDC32-AP2 interactions. Overall, our data demonstrates the function and molecular mechanism of a novel endocytic accessory protein, CCDC32, in CME regulation.

P1088/B90

The Role of Septins In Non-viral Gene Delivery.

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Septin 9 (SEPT9) is a member of a family of small GTPases that modulate intracellular trafficking of vesicles. SEPT9 serves as a scaffold for recruiting dynein motor proteins to late endosomes (LEs) and lysosomes. Intracellular trafficking of LEs and lysosomes is essential for cells' well-being under stress conditions. We sought to understand the role of SEPT9 in preventing the formation of mutant huntingtin (mHTT) aggregates in mouse cells. Surprisingly, our results have shown that the downregulation of SEPT9 via RNA interference in MC3T3 cells affects the expression of genes transiently transfected with lipofectamine. While initial results suggest that SEPT9 siRNA slightly affects the uptake of substrates from extracellular spaces, much of the impact of SEPT9 siRNA appears to be downstream of endocytosis. We are currently exploring possible effects of SEPT9 knockdown (KD) on endosomal escape of transiently transfected genes, gene delivery to the nucleus, transcription, and/or mRNA decay. Overall, this study aims to elucidate the aspect(s) of gene delivery that are more impacted by SEPT9, with possible implications for viral infections.

P1089/B91

EYA protein complex is required for Wntless retrograde trafficking from endosomes to Golgi.

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Retrograde transport of WLS (Wntless) from endosomes to trans-Golgi network (TGN) is required for efficient Wnt secretion during development. However, the molecular players connecting endosomes to TGN during WLS trafficking are limited. We identified a role for Eyes Absent (EYA) proteins during retrograde trafficking of WLS to TGN in human cell lines. By using worm, fly, and zebrafish models, we found that the EYA-secretory carrier-associated membrane protein 3 (SCAMP3) axis is evolved in vertebrates. EYAs form a complex and interact with retromer on early endosomes. Retromer-bound EYA complex recruits SCAMP3 to endosomes, which is necessary for the fusion of WLS-containing endosomes to TGN. Loss of EYA complex or SCAMP3 leads to defective transport of WLS to TGN and failed Wnt secretion. EYA mutations found in patients with hearing loss form a dysfunctional EYA-retromer complex that fails to activate Wnt signalling. These findings identify the EYA complex as a component of retrograde trafficking of WLS from the endosome to TGN.

P1090/B92

Phosphoglycerate kinase 1 cooperates with AP-2 complex to promote endocytosis.

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Coat adaptor proteins are crucial for protein transport, facilitating cargo sorting and membrane deformation. Adaptor protein 2 (AP-2) complex-mediated endocytosis is a key route for cargo receptors endocytosis, such as transferrin receptor (TfR) and epidermal growth factor receptor (EGFR), from the plasma membrane to early endosomes. AP-2 complex as the inner coat adaptor recognizes cargo proteins and recruits clathrin as the outer coat to initiate the vesicle formation. However, failure to entirely block receptor internalization in AP-2-depletion cells suggests the involvement of additional factors in protein endocytosis. Glycolytic enzyme phosphoglycerate kinase 1 (PGK1) has been known to promote EGFR lysosomal transport. Here, we further discovered that PGK1, coupled with AP-2 complex, regulates protein endocytosis. We demonstrated more significant defects in TfR and EGFR endocytosis in PGK1 and AP-2 co-knockdown cells compared with single knockdown cells. We further found that PGK1, behaving like AP-2 complex, also recognized the sorting signal on the cytoplasmic tail of TfR and EGFR, indicating that PGK1 can be the co-adaptor with AP-2 complex to promote cargo sorting during protein endocytosis. These findings broaden the knowledge of AP-2-mediate endocytosis at the molecular level and pinpoint the moonlighting function of glycolytic enzyme PGK1 during the vesicle formation process.

P1091/B93

Dynamins play a novel role in promoting initial invagination of flat clathrin-coated structures.

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Clathrin-mediated endocytosis (CME) plays an essential role in selective uptake of extracellular molecules and plasma membrane components in eukaryotic cells. The formation of an endocytic vesicle is a complex process operated by multiple proteins, including dynamins, which are best-known for their function in mediating scission of deeply invaginated clathrin-coated structures (CCSs) from the plasma membrane in cells. However, dynamins were also proposed to function at earlier CME stages, but their specific role in this capacity remained undefined. Here, we report a novel role of dynamins in promoting the initial invagination of flat CCSs besides their role in the CCS scission. Using immunogold platinum replica electron microscopy (PREM), we found that dynamin2 (a ubiquitous and predominant dynamin isoform in mammalian cells) associates not only with spherical CCSs, as expected given its role in scission, but also with flat CCSs, where it forms linear patterns across the CCS surface, as if dividing it into sub-compartments. Such localization of dynamin2 may reflect its role at earlier stages of CME. Using PREM, we discovered that either knockdown (KD) of dynamin2 in HeLa cells by lentiviral shRNA or inducible triple knockout (TKO) of all three dynamins in mouse embryo fibroblasts, dramatically decreased the fraction of the dome-shaped CCSs (at an intermediate stage of CCS invagination) as compared with control cells containing endogenous dynamins. Instead, these KD or TKO cells contained an increased fraction of small spherical CCSs, apparently stalled at the late stage just before scission, which is consistent with previous studies from various labs. Unexpectedly, the dynamin-deficient cells also contained a significant population of large flat CCSs that apparently failed to invaginate. Thus, our study provides evidence for a novel role of dynamins in the invagination of flat CCSs, which likely corresponds to the hypothesized roles of dynamins in early CME.

P1092/B94

ESCRTs are necessary for micropexophagy in the methylotrophic yeast *Komagataella phaffii*.

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Autophagy, an intracellular degradation system, includes macroautophagy and microautophagy. Macroautophagy involves autophagosome that engulfs and transports the target components to be degraded to the vacuole/lysosome. In contrast, microautophagy is a pathway in which the membrane of the vacuole/lysosome or endosome is directly deformed to incorporate the target components. Compared with macroautophagy, a unified view of the molecular mechanisms of microautophagy has not yet been elucidated. Recently, our laboratory has revealed that Endosomal Sorting Complex Required for Transport proteins (ESCRTs) are necessary for microautophagy of lipid droplets, known as microlipophagy, in the budding yeast *Saccharomyces cerevisiae*. In this study, we aimed to disclose the role of ESCRTs for microautophagy of peroxisomes, referred to as micropexophagy, using the methylotrophic yeast *Komagataella phaffii*. Originally, ESCRTs were identified to be involved in various membrane deformations. We generated disruption strains of *VPS27*, *SNF7* and *VPS4*, genes encoding ESCRTs in *K. phaffii* and examined the morphology of vacuolar membrane during induction of micropexophagy. We found that these mutant strains did not show the changes in the vacuolar morphology as observed in the wild type strain. On the other hand, macropexophagy progressed in these mutant strains, similar to the wild-type strain, indicating that the ESCRTs are required for

micropexophagy, but not for macropexophagy. In conclusion, our results demonstrate that ESCRTs are essential factors for micropexophagy.

P1093/B95

The Small GTPase Arl4A Mediates Exosome Biogenesis via the Regulation of Syntenin.

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Exosomes are a subtype of extracellular vesicles whose release is associated with the fusion of multivesicular bodies (MVBs) with the plasma membrane in cells. Previous studies have shown that the CD63-syntenin-ALIX axis plays an important role in regulating exosome biogenesis in a partially ESCRT-dependent manner. ADP-ribosylation factor (Arf)-like protein 4 A (Arl4A) localizes at the multiple cellular compartments such as the plasma membrane, the trans-Golgi network and endosomal compartments, where it plays roles in mediating cytoskeletal remodeling, cell migration, Golgi integrity and EGFR sorting. In this study, we report that Arl4A mediates exosome biogenesis by regulating the assembly of CD63-syntenin-ALIX axis. We first show that Arl4A interacts with syntenin both *in vitro* and *in vivo*. When purifying extracellular vesicles by ultracentrifugation, we found that knocking down Arl4A reduced the amount of exosome secretion. Interaction sites screening revealed that Arl4A binds to syntenin via its PDZ1 domain and C-terminus, which are also the interaction regions of syntenin with the exosome-associated tetraspanin CD63. We showed that Arl4A can form a complex with syntenin and CD63, and that depletion of Arl4A drives intraluminal budding of syntenin but not CD63 in Rab5a-Q79L-enlarged endosomes. We also observed that Arl4A controls the colocalization of syntenin with the lysosomal marker Lamp1, which may underlie the mechanism of how Arl4A regulates exosome biogenesis via the CD63-syntenin-ALIX pathway. Altogether, we propose that Arl4A controls exosome secretion by regulating the endosomal transport of the exosome protein syntenin.

P1094/B96

Slow Axonal Transport of Clathrin Coated Vesicles.

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Proteins and other macromolecules are conveyed in axons and synapses via fast and slow axonal transport, moving at vastly different overall rates. Previous pulse-chase radiolabeling studies have shown that the composition of these two rate-classes is also different. While fast transport carries vesicles and mitochondria, cytoskeletal and soluble (or cytosolic) proteins are conveyed in slow axonal transport. Here we show that surprisingly, clathrin – a protein known to move in slow axonal transport – is conveyed as vesicles that also contain other endocytic proteins such as AP-2 and FCHO, as well as multiple Rab proteins. The biogenesis of clathrin transport-carriers depends on an intact ER-Golgi network, and the transport of individual particles resemble a stop-and-go motion that has been seen with cytoskeletal polymers moving in slow transport. We also developed a RUSH (Retention Using Selective Hooks) assay to trap clathrin-carriers or synaptotagmin-1-vesicles in the neuronal soma, and then release them into the axon at defined timepoints – confirming the slow overall movement of clathrin, and identifying specific Rab proteins that mediate this movement. Overall, our studies challenge the dogma that vesicles can only move in fast axonal transport, and mechanistically define a unique cargo complex conveyed in slow axonal transport.

P1095/B97

Cargo Identification and Regulatory Mechanisms of Endolysosome Exocytosis in Adipocytes.

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Adipose tissue is a secretory organ that regulates whole body homeostasis. It is estimated that ten percent of the adipocyte secretome under basal state is comprised of unconventional protein secretion (UcPS) cargos that lack signal peptides. Numerous UcPS pathways are associated with pathophysiological settings like inflammation, diabetes, and cancer. However, the exact UcPS pathways these cargos exit, their identities and how they get sorted into specific organelles are largely unknown. One of the first well characterized UcPS cargo in adipocytes is Fatty acid binding protein 4 (FABP4), which leads to insulin resistance. FABP4 is secreted via endolysosomal pathway and its secretion can be induced by forskolin (FSK) and inhibited by chloroquine (CQ). We hypothesized that there are more cargos using this route to get secreted in adipocytes. To identify these cargos, we performed silver stain-MS and got 20 candidates, including proteins from glycolysis pathway and 14-3-3 protein family. Interestingly, these cargos show a brown adipocyte specificity, suggesting a higher basal level of lysosome exocytosis in brown adipocytes. RNA-seq data suggests that higher basal expression of Syt7 in brown adipocytes compared to white adipocytes may contribute to the specificity. Mechanistically, UcPS cargos get sorted into organelles either through membrane remodeling or a translocator. By FABP4-DHFR fusion experiment, I discovered that FABP4's entry into endolysosomal pathway does not require complete linearization, suggesting that FABP4 may get sorted via a membrane remodeling mechanism.

P1096/B98

The RhoGAP Sac7 Negatively Regulates Cathrin-Independent Endocytosis by Restricting Plasma Membrane Rho1 Activity.

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Although most cell types use both clathrin-mediated (CME) and clathrin-independent (CIE) endocytic pathways, yeast were long thought to rely solely upon CME for internalization of plasma membrane cargo. We previously identified the first yeast CIE pathway, which requires the cell wall stress sensor Mid2, the Rho1 guanine nucleotide exchange factors (GEFs) Rom1 and Rom2, the small GTPase Rho1, and the formin Bni1 that generates unbranched actin filaments. While GEFs such as Rom1 activate Rho1 by promoting exchange of GDP for GTP, GTPase-activating proteins (GAPs) are a second family of regulatory proteins that inactivate Rho1 by stimulating GTP hydrolysis. Yeast express four Rho1 GAPs (Bag7, Bem2, Lrg1, and Sac7), which might influence CIE by working in opposition to Rom1/2. Here, we show that deletion of *SAC7*, but not any of the other Rho1 GAPs, restores endocytosis in CME-defective cells. Importantly, *sac7*Δ does not restore endocytosis in CME-deficient cells lacking the Rho1 effector *BNI1*, which is required for the CIE pathway. In live cells, Bem2 and Lrg1 show polarized distribution at the bud tip and bud neck, while Sac7 primarily localizes to the mother cell cortex. We find that Sac7 spatially restricts Rho1 activity at the plasma membrane, where *sac7*Δ cells show increased Rho1 activation, decreased polarization of active Rho1, and Bni1 retention at the cell cortex, which may enhance actin polymerization needed for internalization. Taken together, our results indicate that Sac7 is a negative regulator of CIE in yeast.

P1097/B99

Surface delivery quantification reveals distinct trafficking efficiencies among clustered protocadherin isoforms.

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Plasma membrane proteins transmit molecules and signals between the cell and its environment and enable cells to sense and respond to their surroundings. Clustered protocadherins (cPCDHs) are a large family of cell surface adhesion proteins important for neuronal self- and non-self recognition during brain development. The combinatorial diversity of their expression patterns and their involvement in neuronal circuit formation, dendrite complexity, and synapse formation make cPCDHs intriguing candidate molecules for specifying neuronal connectivity. However, investigations of cPCDH subcellular localization in both neurons and heterologous cells have found cPCDHs predominantly in intracellular compartments rather than on the cell surface. Therefore, a key aspect of understanding cPCDH-mediated cell recognition is determining how cPCDH surface trafficking is regulated. We assessed the surface localization of several cPCDH isoforms and engineered variants, and we developed quantitative metrics to enable comparisons of how well different variants traffic to the cell surface. These comparisons revealed that individual cPCDH isoforms are surface delivered to different extents and highlight the inhibitory role of the α-PCDH EC6 domain in surface trafficking. Our experiments also provide new evidence that contradicts the long-standing assumption that *cis* dimerization controls cPCDH surface delivery, prompting a shift towards considering alternative surface trafficking regulation mechanisms. Beyond cPCDHs, this work establishes a generalizable framework for screening proteins and variants of interest for proper cell surface localization.

P1098/B100

Investigating Collagen Export from the Endoplasmic Reticulum Through an In Vitro Vesicle Formation Assay.

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In eukaryotic cells, the endoplasmic reticulum (ER) serves as the starting point of the secretory pathway, where cargo proteins are generally captured by the coat protein complex II (COPII) for ER export. Procollagen, which has a size over 300 nm and high rigidity, poses a distinct challenge, as it exceeds the conventional 70 nm diameter of COPII vesicles. How ER-derived vesicles enlarge into “megavesicles” to accommodate procollagen remains largely unclear. In this study, we employed an in vitro vesicle formation assay to reconstitute the release of procollagen I (PC1) into transport vesicles. Our findings reveal that the inner COPII components as the sole cytosolic components are sufficient to facilitate the encapsulation of PC1 from the ER. In contrast, disruption of the outer COPII coat enhances PC1 secretion. Through quantitative mass spectrometry, we identified specific transmembrane cellular factors co-enriched with procollagen in transport carriers. Notably, the ER-resident protein VAPA was found to promote the disassembly of the inner COPII coats and inhibit collagen secretion, whereas the cis-Golgi located protein, giantin, was shown to promote the ER export and secretion of PC1. These insights provide a deeper understanding of the mechanisms underlying collagen trafficking and indicates that the vesicle formation assay is a potent tool to identify novel factors involved in collagen secretion.

P1099/B101

Acute Knockdown of Lrrk2 Increases Synaptic-Vesicle Size and Augments Exocytosis.

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Heterozygous *LRRK2* mutations are the most common genetic cause of Parkinson's disease (PD), generally leading to an increase in the kinase activity of LRRK2. Clarifying the normal functions of LRRK2 is not only important to appreciate the context in which the mutant allele confers disease, but is also relevant from a therapeutic perspective, where ongoing clinical efforts are aiming to reduce LRRK2 levels. Previous studies have suggested physiologic roles of LRRK2 at synapses, however, data from germline *Lrrk2* knockout mice have been confounded by compensation, and few studies have systematically examined the role of LRRK2 in a mammalian synapse. Here we used CRISPR/Cas9 technology to acutely deplete endogenous Lrrk2 in cultured mouse hippocampal neurons without affecting levels of Lrrk1, and evaluated synaptic structure and function using electron microscopy and pHluorins - pH-sensitive optical probes that report exo/endocytic synaptic vesicle recycling. Surprisingly, Lrrk2 depletion increased the size of individual synaptic vesicles throughout boutons, and also substantially augmented exocytosis in both quiescent and evoked neurons. These effects are unlikely to be due to inhibition of Lrrk2 kinase activity, because treating neurons with a highly selective kinase inhibitor (MLi-2) did not result in similar phenotypes. Moreover, even a kinase-dead mutant (D2017A) was able to rescue the augmented exocytosis upon Lrrk2 knockdown, further suggesting that the synaptic phenotypes were not related to kinase activity. Our data advocate a structural, non-enzymatic role of Lrrk2 in modulating membranes at synapses and provide new insights into its physiologic role.

P1100/B102

CNG Channel Trafficking to the Rod Outer Segment is Dependent upon Peripherin-2.

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Human vision begins in the retina where light is captured and transduced into an electrical signal within the outer segment organelle of photoreceptor cells. The outer segment is a modified primary cilium built with hundreds of flattened membranous discs that maximize the light-absorbing surface. In rod photoreceptors, disc membranes are physically separate from the plasma membrane surrounding them. To understand how proteins are specifically delivered to the plasma membrane, we focused on the trafficking requirements of the cyclic nucleotide-gated (CNG) channel. It was previously reported that the CNGβ1 subunit is absent from rods in peripherin-2 (Prph2) knockout mice. However, Prph2 is a disc membrane protein found in a tetrameric complex with Rom-1. In this study, we investigate how Prph2 is engaged in CNG channel delivery to the outer segment. Using an *in vivo* electroporation approach, we overexpressed a MYC-tagged CNGβ1 in *Prph2*$^{-/-}$ rods and found it was trapped in internal membranes, but CNGβ1 localization was restored to the outer segment when full-length FLAG-tagged Prph2 was expressed. These data are consistent with a trafficking defect and not protein instability. Membrane proteins utilize two distinct trafficking routes to reach the outer segment: the CNG channel traffics through the Golgi, while the majority of Prph2 bypasses the Golgi although a small fraction in complex with Rom-1 travels through the Golgi. To determine whether the CNG channel is hitching a ride with the Prph2/Rom1 complex, we stained *Rom1*$^{-/-}$ retinas and found the CNG channel was properly localized in the outer segment. Therefore, the Prph2/Rom-1 complex is not required for CNGβ1 trafficking and suggests CNGβ1 and Prph2 are engaged before the pathways segregate. This led us to investigate which

molecular feature of Prph2 is required for CNGβ1 delivery. We expressed Prph2 chimeras containing either the Prph2 N-terminus, tetraspanin core, or C-terminus and showed that the Prph2 N-terminus is necessary to traffic CNGβ1 to the outer segment.

P1101/B103

Identification of a novel and conserved patch in mu1B with implications for AP-1B function in epithelial cells.

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Epithelial cells are crucial for organ development and tissue homeostasis. Frequently, organs are lined with columnar epithelial cells that polarize their plasma membranes into apical domains facing the luminal side and basolateral domains facing neighboring cells and the basal lamina. To maintain this polarity, it is crucial that plasma membrane proteins are sorted correctly to their target locations. Correct sorting is aided by the heterotetrameric clathrin adaptor complex AP-1 which is known for facilitating cargo transport between the TGN and endosomes and for facilitating trafficking to the basolateral plasma membrane. Columnar epithelial cells express two AP-1 isoforms, the ubiquitously expressed AP-1A and the tissue-specific AP-1B. These complexes differ only in the incorporation of their mu1A or mu1B subunits, which are ~80% similar on the amino acid level. Despite this similarity, AP-1B exclusively localizes in recycling endosomes, where it triggers the formation of a PI(3,4,5)P3-positive basolateral sorting platform. In our published work, we defined a conserved patch of three amino acid residues ('loca' patch) in mu1B that was crucial for AP-1B's localization and function in recycling endosomes (Fields et al., MBoC, 2010). Our current work identified a second patch of conserved amino acids ('sky' patch) within the C-terminus of mu1. This patch lies in a region that becomes exposed to the cytosol when AP-1 is membrane-bound. Using 3D confocal microscopy and analysis, we found that both the 'loca' and 'sky' patches control intracellular localization with respect to TGN marker proteins GCC1 and TGN46 as well as other aspects of AP-1 function. Importantly, comparison of non-polarized versus polarized cells revealed that both AP-1A and AP-1B localization changed upon polarization with both complexes showing more separation from the TGN in polarized cells. Taken together, we conclude that AP-1 complexes not only serve to select cargos into nascent clathrin coated-vesicles but also dynamically regulate intracellular organelles.

P1102/B104

Membrane Tension Regulation is Required for Plasma Membrane Repair.

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Disruptions of the eukaryotic plasma membrane due to chemical and mechanical challenges, such as pathogenic attack and shear stress, are frequent and detrimental. These ruptures need to be repaired immediately to maintain proper cell function and avoid cell death. Impaired plasma membrane repair has been further associated with various disease pathologies. However, the cellular mechanisms involved in wound resealing and restoration of homeostasis are diverse and contended. Here, we show that clathrin-mediated endocytosis is upregulated at later stages of plasma membrane wound repair following the actual resealing of the wound in human endothelial cells. Using two-photon laser ablation

assays, we observe that this compensatory endocytosis occurs near the wound, predominantly at sites of previous early endosome exocytosis which is required in the initial stage of membrane resealing, suggesting a spatio-temporal co-ordination of exo- and endocytosis during wound repair. Using cytoskeletal alterations, modulation of membrane tension by hydrogel matrices, membrane area modulations using intercalating agents, and by varying mechanosensitive proteins, we identify membrane tension as a major regulator of the wounding-associated exo- and endocytic events that mediate efficient wound repair. Thus, membrane tension changes act as a universal trigger, apart from the well-established extracellular Ca^{2+} influx, for plasma membrane repair by modulating the exocytosis of early endosomes required for resealing and subsequent clathrin-mediated endocytosis acting at later stages to restore cell homeostasis and function.

P1103/B105

A Novel Microscopy Fusion Assay Reveals That Transition From Membrane Tethering To Fusion Is Modulated By Physical Coupling Between Rab And SNARE Machineries.

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A key concept in intracellular trafficking is targeting and fusion of a cargo-containing vesicle with its correct compartment. This process relies on precise coordination between Rab and SNARE machineries. Rab GTPases, along with their effectors, set compartment identity. SNARE proteins, located on the target (t-SNAREs) and vesicle (v-SNAREs) membranes, assemble into a trans-SNARE complex, providing the energy for membrane fusion. The process includes the following steps: 1) vesicle capture, mediated by long-range tethering effectors (80-200nm) that bind active GTPases, 2) handover to short-range tethering complexes, 3) SNARE priming and membrane docking, modulated by Sec1/Munc18 (SM) proteins, and 4) mixing and fusion of the lipid bilayers. While step 4 is an irreversible process, vesicle in steps 1-3 still has the chance to detach and leave. This raises the question of which factors increase the likelihood of a successful fusion event. *In vitro* fusion bulk assays have advanced our understanding of the molecular players involved in the process, but a comprehensive tool to mechanistically dissect the coordination between the mentioned steps is still missing. In this study, we developed a novel *in vitro* microscopy assay to monitor transition from long-range tethering to fusion, in real-time and at single-vesicle resolution. Using the early endosome fusion machinery as a model system, we discovered that Rabenosyn-5 in complex with the SM protein Vps45 (RV complex) is sufficient to tether vesicles carrying active Rab5. RV tethers membranes more closely and stably than the long-range endosomal tether EEA1, positioning membranes in a pre-docking state. Increasing the concentration of RV complex facilitated the transition from membrane tethering to fusion, suggesting effector concentration on the membrane controls fusion probability. Interestingly, when Rabenosyn-5 was removed from the reaction, efficiency of tethering-to-fusion transition was significantly reduced, even at higher non physiological concentrations of Vps45. This indicates that physical coupling of the SM protein with the short-range tethering effector is necessary for a successful fusion event. Effector concentration inside cells is modulated by Rab domain formation, which may increase the likelihood of productive versus abortive fusion events. Additionally, coupling of the SNARE primer with the short-range tether provides an extra selection checkpoint, ensuring SNAREs engagement occurs only when vesicles have reached the pre-docking stage. This mechanism ensures spatio-temporal coordination of Rab and SNARE activities, and is a core part of the process that guarantees vesicle identity is specifically and efficiently proofread before fusion.

P1104/B106

Uncovering the Role of Retromer and Sorting Nexins in the Unconventional Secretion of CFTR.

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Secretory and plasma membrane proteins are typically transported through the endoplasmic reticulum (ER) and Golgi apparatus before reaching the plasma membrane or extracellular space. However, recent studies have shown that certain proteins can reach their destination via alternative pathways, collectively referred to as Unconventional Protein Secretion (UcPS). Some membrane proteins, in particular, can bypass the Golgi apparatus after synthesis in the ER and utilize these unconventional routes. One such protein is the cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel expressed on the apical membrane of epithelial cells, identified as a cargo protein in the UcPS pathway. Despite significant progress in elucidating the mechanisms and factors involved in UcPS, many aspects of this process remain unresolved. In this study, we employed a CRISPR-Cas9 screening system with a specifically designed sgRNA library to identify key factors regulating UcPS. Our results revealed that components of the retromer complex are crucial for the UcPS of CFTR. Gene knockdown experiments using siRNAs targeting individual retromer components resulted in a decrease in CFTR UcPS. Further investigation demonstrated that sorting nexins (SNXs), which act in coordination with the retromer complex, also contribute to this process. Specifically, we discovered that a particular sorting nexin interacts directly with CFTR, mediating the interaction between the retromer complex and CFTR. This interaction is enhanced under conditions that activate UcPS, such as an ER-to-Golgi blockade, highlighting the critical role of this mechanism in UcPS. These findings suggest that, in addition to their established roles in endocytosis and recycling pathways, the retromer complex and SNXs may have broader functions in unconventional protein trafficking and secretion.

P1105/B107

Understanding the molecular landscape of synaptic vesicles using cryo-electron tomography.

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Chemical synapses mediate much of the intercellular communications in the nervous system through Ca2+-activated release of neurotransmitters from the synaptic vesicles (SVs) into the synaptic cleft and the subsequent activation of postsynaptic receptors and signaling pathways. In situ cryo-electron tomography (cryoET) has provided valuable insights into this pathway via nanometer-resolution tomograms of intact synapses in the brain. However, due to the challenges in sample preparation and cryoET data collection, these datasets have been limited in size and resolution. Thus these datasets do not capture structure and organization of key players like neurotransmitter transporters, V-ATPases, receptors, membrane fission/fusion factors and how they contribute to overall synapse function. To close this knowledge gap, we are developing a high-throughput cryo-electron tomography (cryo-ET) pipeline to study the molecular landscape of synapses via synaptosomes. Synaptosomes are isolated neuronal tissue fragments containing functional and structurally intact synaptic components. Here, we will present our study of synaptosomes purified from the hippocampus of the rat brain. We have acquired 1000 tomograms of synaptosomes for which we are developing automated pipelines for the identification and segmentation of the synaptic vesicles. Using this pipeline we will carry out a statistical

analysis of the distribution of synaptic vesicles and how they are pooled in the presynaptic terminal via their connections to each other and to the plasma membrane. We will also classify the transmembrane protein complexes in synaptic vesicles using 2D classification and 3D refinement. With this approach we will quantify the distribution of V-ATPases and neurotransmitter transporters across synaptic vesicles. Our work will deliver new insights into synapse organization and function and how it is perturbed in neurodegenerative diseases. This dataset will be available on the CZII CryoET Data Portal (https://cryoetdataportal.czscience.com/).

P1106/B108

Regulation of actin-mediated endocytosis by yeast profilin Pfy1p.

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Clathrin mediated endocytosis is the process by which cells internalize extracellular materials, cell surface proteins, and lipids via clathrin coated vesicles (CCVs) formed by membrane invagination. Internalized CCVs are first delivered to sorting compartments and then recycled back to the plasma membrane or brought to late endosomal compartments en route to the lysosome/vacuole for degradation. We previously showed that transport of CCVs is mediated by actin filament. We also found that *SRV*2, monomeric actin binding protein, regulates actin polymerization and de-polymerization during formation of CCVs. Srv2p binds actin de-polymerization factor Cof1p and actin polymerization factor Pfy1p and these proteins coordinately regulate rapid turnover of actin filaments. Pfy1p binds to ADP-G-actin and catalyze nucleotide exchange of ADP to ATP-G-actin. To further elucidate actin-mediated regulation in CCV formation and transportation, we isolated *pfy1* temperature-sensitive (ts) mutant cell (*pfy1-3* and *pfy1-5*) by PCR-based random mutagenesis. We found that both mutants exhibit abnormal actin patch dynamics, and have defect in actin assembly at endocytic sites, resulting in defective internalization of CCVs. Interestingly, *pfy1-3* mutant cells exhibited remarkable delay in actin polymerization,whereas the *pfy1-5* cells had defects in formation of long actin filaments responsible for CCVs transport. In addition, we observed rod like aggregates of Cof1p in *pfy1-5* mutants but not in *pfy1-3* mutant cells. These results suggest that profilin cooperate with Srv2/CAP and Cofilin to regulate rapid actin assembly during endocytosis and regulates actin-mediated CCV internalization.

P1107/B109

Mechanism of Intracellular ANGPTL3 and ANGPTL8 Trafficking.

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Lipids circulate in the blood in lipoproteins including chylomicrons and very low-density lipoproteins (VLDLs). Lipoprotein Lipase (LPL) is the main enzyme that hydrolyzes the triglycerides from circulating lipoproteins into free fatty acids that can be taken up by cells. Without LPL, dangerously high levels of lipoproteins circulate in the blood, which can lead to cardiovascular disease. LPL inhibitors, known as angiopoietin-like (ANGPTL) proteins, have key roles in the regulation of lipid metabolism. ANGPTL3 is a potent inhibitor of LPL. ANGPTL8 can form a complex with ANGPTL3 which results in more efficient secretion of ANGPTL8 and greater inhibition of LPL than ANGPTL3 alone. Based on immunofluorescence staining of LAMP1 (lysosomal-associated membrane protein 1), I have found that ANGPTL8 when expressed alone, gets trafficked toward lysosomes. However, when ANGPTL8 is co-expressed with

ANGPTL3, both ANGPTL3 and ANGPTL8 are effectively trafficked out of the cell. Using the Retention Using Selective Hooks (RUSH) assay, I confirmed that a population of ANGPTL8, when expressed alone, gets trafficked to lysosomes. I have also found that ANGPTL3 and ANGPTL8 are synthesized and form a complex in the endoplasmic reticulum (ER) and get trafficked out of the cell together. Thus, ANGPTL3 diverts a pool of ANGPTL8 from lysosomal degradation. Future goals include understanding why ANGPTL8 is unstable when it is not in complex with ANGPTL3, as well as visualizing the trafficking dynamics of the ANGPTL3/8 complex.

P1108/B110

Exploring weak multivalent interactions formed by the EH domain proteins of the endocytic machinery in *Saccharomyces cerevisiae*.

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Clathrin-mediated endocytosis (CME) is spatially and temporally regulated by a choreographed interaction of over 50 proteins though the precise mechanisms of this intricate network remain poorly understood. In *Saccharomyces cerevisiae*, the early phase of CME is variable in duration (1-3 min). During this time, the early proteins cluster at the plasma membrane and define the endocytic site. Upon the recruitment of the late coat proteins and the actin regulators, the endocytic pathway becomes regular (30-35 sec).The mechanism for the transition from variable early to regular late phase is still an open question in the field. We aim to understand the role of weak multivalent interactions formed by the clathrin adaptors and endocytic scaffolds in regulation of spatiotemporal dynamics of endocytosis.Recently, one of the early arriving yeast endocytic proteins, Ede1, was shown to form liquid condensates upon over-expression. The ability of Ede1 to form condensates is proposed to promote the initiation of endocytosis. To explore the possibility of other proteins forming condensates, we performed an over-expression screen of multiple endocytic proteins. While most of the proteins were dispersed in cytoplasm in higher concentration, one of the late coat proteins, Pan1, formed condensates. Pan1 is an EH domain protein which shares structural similarities with Ede1. Pan1 condensates exhibited liquid like properties such as fast exchange of molecules, and sensitivity to temperature changes or hexanediol treatment. Electron microscopy revealed Pan1 condensates as a ribosome exclusion zones devoid of membranes. The Pan1 condensates specifically recruited late endocytic proteins like Abp1, Sla1 which might reflect Pan1's function at endocytic sites. We hypothesize that the condensation properties of Pan1 give flexibility to the endocytic machinery to allow dynamic rearrangements of the proteins for successful endocytic event.Ede1 and Pan1 can form weak multivalent interactions via their EH domains with conserved NPF motifs across clathrin adaptor proteins. These weak multivalent interactions may be driving phase separation of Ede1 and Pan1 at the endocytic sites. This interaction is thought to be regulating the transition from early to late phase. Upon precise disruption of these multivalent interaction by mutating 16 NPFs of the four clathrin adaptors, contrary to the prevailing view, we found that the late phase along with the actin polymerisation remains unperturbed. We aim to assess the possibility of EH domains regulating other aspects of endocytosis beyond forming weak multivalent interactions by specifically deleting the EH domains.

P1109/B111

RAB7A phosphorylation at Serine 72 (S72) by NEK7 modulates retromer recruitment to late endosomes.

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Soluble lysosomal proteins form complexes with sorting receptors such as the mannose 6-phosphate receptor and sortilin in the trans-Golgi Network (TGN) to ensure their delivery to the endolysosomal compartment. Once the receptor-cargo complex reaches the endosome, a decrease in pH releases the cargo, and the receptor is retrieved back to the TGN for another round of sorting. The retrieval of receptors is at least in part regulated by retromer, which must be recruited to endosomal membranes, a process that requires the small GTPase Rab7A. Previous work from our group has shown that post-translational modifications (PTMs) such as palmitoylation of Rab7A is required for retromer recruitment. This finding prompted us to explore whether other PTMs play a role in this process, and if there is an interplay among them that could serve a regulatory function. Work from other groups has shown that phosphorylation on Rab7A serine 72 (S72) can regulate effector interactions and functions, so using direct mutagenesis, we engineered Rab7A mutants targeting this site and assessed its ability to recruit retromer in Rab7A knockout (Rab7A^{KO}) HEK293 cells. Immunofluorescence analysis revealed that a non-phosphorylatable mutant at S72 (Rab7A$_{S72A}$) was unable to recruit retromer. Further investigation using Acyl-RAC, demonstrated a reduction in palmitoylation of Rab7A$_{S72A}$. Since palmitoylation is essential for retromer recruitment, we examined the interaction between Rab7A and retromer, and found a weaker interaction with Rab7A$_{S72A}$ with retromer compared to wild-type Rab7A. To determine which kinase modulates Rab7A S72 phosphorylation, we tested known kinases that have been shown to phosphorylate Rab7A. We compared the Rab7A/retromer interaction and membrane distribution of retromer in TBK1^{KO} and TAK1^{KO} HEK293 cells versus wild-type cells but found no significant differences. However, we identified NEK7 as a novel kinase for Rab7A. In NEK7^{KO} cells, we observed reduced phosphorylation and palmitoylation of Rab7A, a diminished interaction between Rab7A and retromer, along with a decrease in retromer recruitment. This resulted in a weaker retromer/sortilin interaction, and decreased retrieval of the receptor. Our findings suggest that both palmitoylation and phosphorylation of Rab7A are critical for modulating retromer recruitment and the subsequent recycling of sorting receptors back to the TGN, processes essential for proper lysosomal function. We establish NEK7 as the kinase responsible for phosphorylation at S72 in this pathway.

P1110/B112

Antisense oligonucleotide treatment restores the function of a CLN3 mutation involved in Batten disease.

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CLN3 Batten disease is a neurodegenerative, lysosomal disease characterized by an early onset of symptoms (loss of vision, seizures, cognitive decline) in childhood usually leading to premature death. CLN3 is an integral membrane protein localized to endolysosomes, and the most common disease mutation is a deletion of exons 7 and 8 of the *CLN3* gene (CLN3$^{\Delta 7/8}$), which results in a truncated form of

CLN3. There are currently no treatments for this disease, but the treatment of a $CLN3^{\Delta 7/8}$ mouse model using antisense oligonucleotides (ASOs) that induced skipping of exon 5 ($CLN3^{\Delta 5/7/8}$) offers a novel therapeutic approach. This resulted in a longer CLN3 protein, reduced histopathology, improved motor coordination and increased lifespan of ASO treated mice. We have shown that CLN3 interacts with the small GTPAse Rab7A and regulates its ability to recruit the retromer complex affecting the endosome-to-trans Golgi Network (TGN) retrieval of the lysosomal sorting receptors, such as sortilin and mannose 6-phosphate receptor. CLN3 knockout HeLa cells ($CLN3^{KO}$) displayed reduced Rab7A/retromer and retromer/sortilin interactions and decreased lysosomal enzymes activity. Thus, we sought to determine if $CLN3^{\Delta 7/8}$ loses its ability to interact with Rab7 and if ASO corrected versions of CLN3 could restore this interaction and impact downstream functions. We found a loss of interaction between $CLN3^{\Delta 7/8}$ and Rab7, retromer and sortilin, that was recovered with the ASO corrected CLN3 ($CLN3^{\Delta 5/7/8}$ and $CLN3^{\Delta 6/7/8}$). We also found that overexpressing $CLN3^{\Delta 5/7/8}$ and $CLN3^{\Delta 6/7/8}$ in $CLN3^{KO}$ cells rescued the Rab7/retromer interaction whereas only $CLN3^{\Delta 5/7/8}$ was able to rescue the retromer/sortilin interaction. Without this last interaction, the lysosomal sorting receptors remain in endosomes and are eventually degraded in lysosomes, resulting in inefficient sorting of cargo proteins and dysfunctional lysosomes, as observed in CLN3 Batten disease. Finally, we observed increased lysosomal enzyme activity in $CLN3^{\Delta 7/8}$ mice cells treated with the exon5 targeting ASO. Together, these results suggest the $CLN3^{\Delta 7/8}$ mutation leads to a loss of function of the endosome-to-trans Golgi Network (TGN) retrieval of the lysosomal sorting receptors, at least partially explaining Batten disease pathophysiology, which could be treated using ASOs.

P1111/B113

Loss of Function Phenotypes for *Osbpl9* and *Osbpl11* in Leydig Cells Do Not Indicate Involvement in Steroidogenesis.

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Oxysterol-binding protein-like proteins (*Osbpls*) are a large gene family of lipid-transfer proteins characterized by an N-terminus pleckstrin homology domain (PHD) and a C-terminus Osbp-homology domain (OHD). The PHD binds to phosphatidylinositides, which encode organelle identity, to anchor to organelle membranes, while the OHD contains a hydrophobic cavity that binds cholesterol and oxysterols. Leydig cells express 7 different *Osbpls*, of which mRNA expression of *Osbpl9*, *Osbpl8* and *Osbpl11* were among the highest. Function of *Osbpl9* has been proposed to involve the transport of sterols between ER and trans-Golgi network to regulate the early secretory pathway; *Osbpl11* has been proposed to form a heterodimer with *Osbpl9*, together regulating the exchange of phosphatidylserine and phosphatidylinositol-4-phosphate at the ER-Golgi site to regulate synthesis of sphingolipids known for multiple roles in steroidogenic induction. Additionally, a phosphoproteomics study has indicated that *Osbpl11* is phosphorylated in response to steroidogenic signaling in MA-10 Leydig cells. To investigate the roles of *Osbpl9* and *Osbpl11* in steroidogenesis, we used CRISPR-Cas9-mediated gene targeting in MA-10 Leydig cells. We deleted the OHD for *Osbpl9*, targeting exons 15-17, across the 19 possible splice variants, and completely abrogated *Osbpl11*, which lacks splice variants, by targeting exon 1. The *Osbpl9* and *Osbpl11* knockout clones were validated by PCR screens and sequencing of the targeted genomic loci. Functional analysis of steroidogenesis in *Osbpl9* and *Osbpl11* knockout clones, as measured by their response to hCG-induced progesterone biosynthesis, revealed no significant differences in steroid production compared to wild-type cells. These findings suggest that *Osbpl9* and *Osbpl11* are dispensable

for steroidogenic induction in Leydig cells, and the possible presence of functionally redundant mechanisms compensating for their loss.

Cell Adhesion and Communication 1

P1112/B114

High Glucose Impairment of Epithelial Barrier Properties Correlates with Claudin Isoform Expression.

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Diabetes and hyperglycemia impair the homeostasis of vascular and epithelial cells. We previously identified pathways of cell-cell communication between endothelial and epithelial cells, mediated by Semaphorins (ligands) and Plexins (receptors), which are affected by diabetes. The epithelial/endothelial layers are a functional unit constituting a selective barrier. How the barrier properties are affected by diabetes remains insufficiently studied. Our objective is to study whether high glucose impairs the epithelial barrier properties by affecting tight junction claudin proteins and interfering with sensitivity to semaphorins. We studied tight junction barrier strength via trans-epithelial electrical resistance (TEER), measured in Calu-3 epithelial cells cultured in trans-well permeable support. We observed that high glucose (25mM) increased TEER over a course of 2 weeks, while a mannitol osmolality control (20mM) had no effect (ANOVA). This strengthening of the epithelial barrier correlated with higher expression of tight junction claudin isoforms 3, 7, 16, 19, 20, 22, 24. In contrast, the pore-forming claudin-2 was decreased. Other claudins (1, 4, 8, 10, 23) were not affected by high glucose. Mannitol did not change any claudin. To explore the role of diabetes, we measured surface Claudin-2 via surface biotinylation in renal epithelial tubules isolated from diabetic mice (Akita), and found it to be decreased by 95 ± 3% (t-test $p<0.01$). Finally, to study sensitivity to semaphorins in Calu-3 cells exposed to high glucose, we treated them with 300ng/ml of Semaphorin-3F (Sema3F), the predominant semaphorin we found secreted by endothelial cells. We observed that Sema3F stimulated the time course of TEER in Calu-3 cells, while this stimulatory effect was absent in cells on high glucose. Intriguingly, we did not observe any decrease in the expression of plexin isoforms, but a significant increase in Plexin-A2 expression. However, intracellular signal transduction was not measured. We conclude that high glucose strengthens the Calu-3 epithelial barrier by favoring expression of tight junction claudins, and decreases sensitivity to Sema3F. These observations, together with lower claudin-2 expression (a pore-forming claudin) is expected to decrease epithelial barrier permeability, potentially contributing to the detrimental effects of diabetes.

P1113/B115

Evolutionary, Biochemical, and Functional Characterization of the GOLPH3 Family of Golgi Apparatus Oncoproteins.

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GOLPH3 is a phosphatidylinositol 4-phosphate (PI4P) binding, peripheral membrane protein of the trans-Golgi network that plays an essential role in vesicle trafficking. It has also been recognized as an oncoprotein. GOLPH3 has a paralog called GOLPH3L, which is also localized at the Golgi apparatus,

although its exact function is not fully understood. Both GOLPH3 and GOLPH3L possess an intrinsically disordered region (IDR) in their N-terminal region, the functional significance of which remains uncertain. Our unpublished research reveals that the gene duplication event leading to GOLPH3 and GOLPH3L originated in the vertebrate ancestor. Consequently, we sought to examine these proteins in invertebrates, where a single-copy gene (GOLPH3/3L) represents the ancestral condition. We found a high degree of amino acid sequence divergence in the N-terminal region of the GOLPH3 family, in contrast to a high degree of conservation in the C-terminal globular structure. Therefore, we decided to characterize the biochemical and functional consequences of these evolutionary variations in this family of oncoproteins. We conducted phylogenetic analysis, fluorescence microscopy, oligomeric analysis, circular dichroism (CD), limited proteolysis, thermal stability, lipid binding, and binding partners analysis of GOLPH3 orthologs from 11 species representing the tree of life from yeast to humans. Despite substantial divergence within their IDRs, all proteins exhibited conserved Golgi localization, although some orthologs also showed distinct additional localization. Oligomeric analysis, CD data, and limited proteolysis indicated a similar globular structure; however, thermal stability analysis revealed remarkable variability. Likewise, lipid binding and binding partners analysis showed intriguing differences. Our findings indicate that divergence in the IDRs of the GOLPH3 family carries distinct structural information that influences their biochemical and cellular properties. Acknowledgments: FONDECYT 1211481.

P1114/B116

A cell non-autonomous signaling program downstream of the Integrated Stress Response adjusts homeostatic capacity.

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A cell non-autonomous signaling program downstream of the Integrated Stress Response adjusts homeostatic capacity Francesca Zappa[1], Julia Conrad[1], Morgane Guzman[1], & Diego Acosta-Alvear[11]Altos Labs Inc. 1300 Island Drive Redwood City, CA 94065

The Integrated Stress Response (ISR) is a central signaling network that allows cells to detect and react to various stresses to restore homeostasis or trigger cell death if the stress is too severe. Both cellular outcomes, adaptation or cell death, benefit the organism and require coordination amongst cells. This coordination may occur via cell-cell communication, allowing cells to share information about their state. Further, one could imagine that such communication maintains homeostasis across biological scales, from cells to tissues, organs, and the organism. To investigate whether ISR signals can be communicated intercellularly, we employed a comprehensive strategy composed of synthetic biology tools, multi-omics, and cell co-cultures consisting of message “sender” cells carrying a synthetic tool for stress-free ISR activation with a small molecule, and message “receiver” cells (i.e., naïve cells). This approach allows us to investigate hard-wired cellular responses without the molecular damage and pleiotropic effects elicited by commonly used supraphysiological perturbagens. Moreover, it enables the activation of the ISR in a defined fraction of a cell population. Our experiments revealed that activating the ISR within the sub-population of senders influenced the response of receiver cells, as evidenced by activation of gene expression programs driving biosynthesis. Consistent with these observations, receiver cells show increased polysome fractions, basal respiration and mitochondrial spare respiratory capacity. We corroborated these findings in human brain organoids exposed to an ISR inducing drug. Single-cell transcriptomes collected from these organoids revealed that astrocytes and neurons induce gene expression profiles reminiscent of those observed in sender and receiver cells, where astrocytes

assume the role of senders and neurons of receivers. To test the hypothesis that secreted factors may mediate this cell-cell communication phenotype, we cultured cells carrying stress response reporters in conditioned media obtained from cell cultures in which we synthetically activated the ISR. These experiments revealed a shift in the activation threshold of stress responses induced by classical ISR-triggering poisons. Together, these findings underscore the transmissibility of the ISR to modulate cellular responses across spatial domains and shed light on the intricate interplay between inter- and intracellular ISR-dependent signaling cascades.

P1115/B117

Multimodal physical protection against immune cell attack by the cancer glycocalyx.

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Cancer is often associated with the aberrant expression of cell-surface mucins, resulting in a thick cellular glycocalyx that coats the cancer cell surface and governs its interactions with surveilling immune cells. Densely packed mucin biopolymers tethered to the plasma membrane can assemble into a nanoscale barrier that protects cells from lysis by immune cells, including engineered immune cells such as CAR-T and CAR-NK cells. Here, we ask whether a bulky cancer glycocalyx can impart physical protection against immune cell attack by disrupting the many dynamic events leading up to cell-mediated cytotoxicity. We utilize a cellular model with tunable expression of the cancer-associated mucin Muc1 to examine the effect of the mucin barrier on the dynamics of interactions with Natural Killer (NK) cells. Live-cell imaging reveals that target cells with a thin mucin barrier allow for a high proportion of stable contacts with NK cells, resulting in rapid lysis of the target within minutes. Meanwhile, target cells with a thick mucin barrier are significantly more likely to resist stable NK cell adhesion, activation, and killing. Strikingly, these target cells are still able to resist continuous engagement and attack for several hours. We also find that killing of high mucin-expressing target cells by an NK cell is characterized by the appearance of apoptotic blebs rather than by lytic pore formation, suggesting the mucin barrier may preferentially protect against perforin pore assembly on the tumor cell membrane. At high surface densities, mucins also cause dramatic bending of the plasma membrane, resulting in exotic membrane shapes that surveilling immune cells must form synapses with. High-resolution imaging of the NK-target contact interface reveals that mucin-mediated plasma membrane curvature alters the nanoscale architecture of the immune synapse, and may therefore prevent efficient receptor organization and clustering at the immune synapse. Taken together, these results suggest that the mucin barrier acts along multiple levels to provide physical resistance to immune cell attack, by (i) hindering stable contact formation, (ii) preventing effector cell activation and polarization, (iii) resisting pore-mediated lysis and (iv) generating dramatic membrane curvature that discourages productive synapse formation.

P1116/B118

Role of Intestinal Epithelial SEPT9 in Regulating Gut Barrier Integrity, Mucosal Inflammation and Colonic Tumorigenesis.

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The intestinal epithelium comprising a single layer of polarized intestinal epithelial cells (IECs) connected by cell-cell junctions- is the body's largest interface with the external environment. This monolayer is the site of nutrient uptake and digestion and defense against pathogens, and its disruption may lead to mucosal inflammation and increased susceptibility to colorectal cancer (CRC). Cytoskeletal components are important regulators of cell-cell junction integrity and epithelial polarity. While the functions of actin, microtubules, and intermediate filaments in these processes are well-documented, the contribution of the filament-forming septin family is poorly understood. This highlights an important knowledge gap since dysregulation of septins, especially septin 9(SEPT9) in humans is linked to CRC pathogenesis.
To address this gap, we investigated the role of SEPT9 in intestinal epithelial homeostasis using SEPT9-NeonGreen knockin mice, inducible IEC-specific *SEPT9* knockout (KO) mice, *SEPT9*-knockout HT-29 human IEC line, SEPT9-interactomics in DLD-1 human IECs, and patient tissue.
We determined in SEPT9-NG mice, and immuno-stained human IEC lines and human intestinal tissue, that SEPT9 accumulates at apical junctions of IECs and colocalizes with tight junction (TJ) proteins ZO1, claudin-3 and non-muscle myosin IIC (NM IIC), all of which were found to be part of the SEPT9 interactome in DLD-1 cells. Loss of SEPT9 in mouse IEC in vivo and human IEC in vitro resulted in barrier disruption, according to dextran-permeability and transepithelial electrical resistance measurements, repectively. IECs lacking SEPT9 showed defects in cell apical morphology, as determined by machine learning-based quantification. Specifically, cells deviated from regular polygonal shapes and displayed junctions with increased tortuosity. Additionally, ZO1, claudin-3 and NM IIC were mis-localized from apical junctions. Consistent with this, SEPT9 KO mice displayed significantly higher susceptible to dextran sodium sulfate-induced colitis and colitis-associated colon cancer, compared to controls.
Our data suggest that SEPT9 is required for the localization of TJ components in polarized IECs to maintain intestinal epithelial barrier integrity under homeostatic conditions and during mucosal inflammation. These findings provide new insights to understanding the link between SEPT9 dysregulation, gut inflammation and CRC.

P1117/B119

Single nucleus RNA-seq reveals a role of PLXNA4 in arrhythmogenic right ventricular cardiomyopathy.

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Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a poorly understood and underdiagnosed heart muscle disease that is marked by replacement of the myocardium with fibro-fatty tissue, ventricular arrhythmias, and sudden death. ARVC is an inherited disease with around 40% of patients presenting with mutations in desmosomal proteins, which are critical in maintaining cell communication and adhesion in cardiomyocytes. Current treatment strategies have had limited success in slowing or reversing the progression of the disease, so increasing our knowledge of the molecular pathways involved in ARVC is critical for improving patient outcomes. To explore the transcriptional mechanisms that drive this disease, single nucleus RNA sequencing (snRNA-seq) was performed on the right ventricle (RV) of 8 ARVC patients and 11 non-failing controls profiling a total of 139,347 nuclei. The transcriptional signature of this disease largely converges with that of ischemic cardiomyopathy (ICM), dilated

cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) datasets previously collected, which suggests that end stage cardiomyopathy shares a common transcriptional pathway. In ARVC, there is a significant decrease in the number cardiomyocytes and increase in the number of adipocytes compared to the three other cardiomyopathies studied. Only 13 genes are uniquely dysregulated in ARVC. One of these genes, PLXNA4, was increased in the cardiomyocytes of ARVC patients compared to non-failing controls (logFC=0.86, P=3.95e-5). PLXNA4 is a receptor in the semaphorin signaling pathway that has previously been implicated in axon guidance and cell motility pathways. Immunofluorescent staining of RV tissue demonstrates that PLXNA4 localizes to the sarcomeres and intercalated discs of cardiomyocytes. In addition, staining confirms the RNA expression pattern of PLXNA4 that was observed across samples in the snRNA-seq data (r=0.824). PLXNA4 overexpression in iPSC-derived cardiomyocytes causes a downregulation of cardiac conduction pathways and an upregulation in immune response pathways suggesting a role in the inflammatory response and arrhythmias in ARVC. Our study provides a comprehensive transcriptional profile of ARVC at the single cell level and provides a novel role of PLXNA4 in disease.

P1118/B120

Skin-axon interactions in zebrafish.

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One important function of the skin is to detect touch and pain. The epithelial keratinocytes of the skin are heavily innervated by sensory axons, which are frequently enclosed by keratinocytes. This phenomenon of '*axon ensheathment*' is conserved across species, including humans. However, the molecular mechanism and functional relevance of ensheathment, as well as the signalling pathways that mediate axon-keratinocyte recognition are poorly characterized. The high degree of conservation with human epidermis, genetic amenability and optical clarity of the zebrafish skin make it a fantastic model to study ensheathment. Previous work reported the enrichment of lipid microdomains and junction proteins at axon-keratinocyte contact sites, suggesting that they may play a role in ensheathment. To better understand the roles of lipids in this process, I will investigate the mechanisms of lipid clustering using photoconversion of lipid reporters, imaging of ER-plasma membrane contact sites, and genetic perturbation of lipid homeostasis. To investigate how junctions contribute to ensheathment, I will create an inducible system to study the role of E-Cadherin in ensheathment. To identify the specific receptor-ligand interactions involved in ensheathment at the axon-skin interface, I will conduct a CRISPR-based knockdown candidate screen of candidate signaling pathways. Finally, I will conduct behavioural assays to test how knocking down ensheathment genes affects nociception. My studies of axon-skin interactions will provide insight into innervation of the epidermis, cell-cell interactions, and somatosensation.

P1119/B121

Exploring the Ly6 Family in C. elegans: Identification and Expression of hot1 and Interaction Studies of hot3.

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The Ly6 family has been extensively studied across various species including Drosophila, C. elegans, mice, and zebrafish. Proteins of the Ly6 family share homology with snake venom alpha neurotoxins that

target with nicotinic acetylcholine receptors (nAChR); previous work has shown that rodent Ly6 proteins can regulate nAChR activity as well, suggesting that the Ly6 family may be of interest for treatment of nAChR-related neurodegenerative diseases . C. elegans have a total of 10 Ly6 encoding hot genes;. to date, only one C. elegans Ly6 protein-odr-2-has been characterized and has been found to be involved in sensory signaling including AWC-mediated olfactory behaviors. Here, we are expanding our knowledge of Ly6 function in C. elegans by investigating the expression and potential interacting partners to hot1 and hot3, respectively. To determine the expression pattern of hot1, we generated worm lines containing a transcriptional GFP reporter for hot1 and the NeuroPAL transgene. NeuroPAL is a transgene that creates color-coded reference maps of the hermaphrodite C. elegans nervous system and is helpful in identifying specific neurons that may be confused with neighboring neurons. Using confocal microscopy, we have identified several hot1-expressing neurons, including DD1, DD2, and DD3. To determine interacting partners for hot3, we introduced an mNeonGreen epitope tag to the N-terminus of hot3 at the genomic locus and have developed a protocol for immunoprecipitating endogenous hot3. We hope to further optimize the assay to increase the efficiency of pulldown in order to eventually identify interacting partners via mass spectrometry.

P1120/B122

The molecular and functional interactions between keratinocytes and nociceptive neurons in the skin.

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The skin is the largest sensory organ and detects stimuli such as temperature, touch, pain, and itch through diverse neurons and receptors. MrgprD (Mas-related G Protein-coupled receptor D) and CGRP (calcitonin gene-related peptide) sensory neurons are two specialized subsets of pain receptors that densely innervate the skin. Their cell bodies reside in the dorsal root ganglion (DRG), with central axons projecting into distinct laminae of the spinal cord. In the skin, the peripheral axons of MrgprD neurons terminate in the granular layer of the epidermis, while CGRP neurons target the spinous layer. This spatial segregation implies that granular and spinous keratinocytes provide specific cues for these neurons to specifically target them and thus to transmit sensory signals to the spinal cord precisely. However, the regulatory mechanisms and functions of these layer-specific interactions between keratinocytes and neurons are not well understood. In this study, we aim to unveil the role of keratinocytes in regulating nerve innervation and sensory transduction for these two types of nociceptive neurons.

P1121/B123

Glia preserve their own functions while compensating for neighboring glial cell dysfunction.

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Glia not only form close associations with neurons throughout the CNS, but they also interact closely with each other. As these cells mature, they undergo glial tiling to abut one another without invading each other's boundaries. Upon the loss of the secreted neurotrophin Spätzle 3 (Spz3), *Drosophila* cortex glia transform morphologically and lose their intricate interactions with neurons and surrounding glial subtypes. Here, we reveal that all neighboring glial cell types (astrocytes, ensheathing glia, and subperineurial glia) react by extending processes into the previous cortex glial territory to compensate

for lost cortex glial function by reducing the buildup of neuronal corpses via Draper-mediated engulfment. However, the loss of Spz3 alone is not sufficient for glia to cross their natural borders, as blocking CNS growth via nutrient-restriction prevented both morphological cortex glial disruption and the aberrant infiltration induced by cortex glial Spz3 depletion. Remarkably, even when astrocytes, ensheathing glia, and SPG divert their cellular resources into the cortex, they are still capable of performing their normal functions such as synaptic remodeling during metamorphosis (astrocytes), post-injury clearance of neurite debris (ensheathing glia), and regulation of the blood brain barrier (SPG) suggesting that in the presence of neighboring glial cell dysfunction, multiple neighboring glial subtypes multitask to continue to preserve their own normal functions to maintain CNS homeostasis.

P1122/B124

CD2AP in endothelial monolayer dynamics.

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The formation of branched actin networks is essential to cell migration, cell junction formation, and endocytosis. Besides actin, branched actin networks require activated Arp2/3 complex and barbed-end capping protein. Arp2/3 complex is activated by membrane associated nucleation promoting factors, such as WASp and WAVE complex. Capping protein (CP) is bound to its inhibitor V-1 (myotrophin) in the cytosol, which is released by membrane-associated proteins containing CP-interacting (CPI) domains. The CPI-containing protein CARMIL1 is localized to the leading edge of migrating cells and is essential for lamellipodium formation and extension; whereas the CPI-containing protein WASHC2C is a subunit of the WASH complex that is essential for endosome formation and tubular fission. In endothelial cells, branched actin networks maintain cell-cell junction morphology, dynamics, and permeability and are required for transcellular trans-endothelial migration (TEM) of immune cells. We have shown that N-WASP is required for junction integrity, whereas WAVE2 is involved in TEM in human microvascular endothelial cells. The specific CPI-containing proteins required for junction integrity and TEM have not been established. CD2AP is a CPI-containing protein that localizes to membrane ruffles and lipid rafts through interaction of its SH3 domains with membrane proteins. CD2AP is expressed in microvascular endothelial cells, including brain endothelial cells, and mutations in CD2AP have been associated with Alzheimer's disease, suggesting an important role in vascular integrity. We are studying the roles of CD2AP in endothelial cells, specifically in regulating cell junction permeability and dynamics and in regulating immune cell trafficking across endothelial monolayers.

P1123/B125

The temporary unilateral common carotid artery occlusion induces BBB dysfunction.

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The blood-brain barrier (BBB) restricts the flow of ions, proteins, and cells to the brain parenchyma to protect neuronal activity. In cerebrovascular diseases, including stroke, BBB breakdown contributes to neuronal death, infarction and cognitive dysfunction. Nevertheless, little is known about the early changes in BBB caused by this ischemic condition. The control of endothelial permeability provides an

option to prevent the signaling cascades that lead to neuronal death. Therefore, this study aimed to determine how BBB disruption is induced by temporary unilateral common carotid artery (CCA) occlusion, an *in vivo* rat model of ischemia-reperfusion (IR). The left CCA of male Wistar rats was exposed and clamped for 2h to interrupt blood flow; then, the clamp was retired to allow natural reperfusion. BBB structure and function changes were determined after 3h, 24h and 7 days of IR. First, we evaluated the areas of infarction by staining metabolic active cells with triphenyltetrazolium chloride; surprisingly, no cell death areas were found. Consistent with this result, immunostaining with caspase-3 antibody did not detect apoptotic cells in ischemic brains. To evaluate changes in vascular coverage induced by IR, brain sections were stained with the vascular marker isolectin B4 and analyzed with the angiogenesis plugin for ImageJ. We found similar vessel length and number of segments in IR rats when we compared them with Sham controls. Then, BBB function was determined by the intravenous injection of Evans blue in Sham or IR rats. We have found increased dye permeability in the right brain hemispheres of IR rats after 24h and 7 days, as compared with the contralateral hemispheres and with Sham. When we analyzed the localization of the tight junction protein claudin-5, importantly, we found decreased expression at the endothelial cell contacts in those vessels with higher permeability, suggesting that Evans blue dye infiltrates into the brain through endothelial junctions. We also evaluated the changes in pericytes, the cells that support BBB function. Consistent with BBB breakdown, decreased association of pericytes to brain vessels was found in brains with IR. Together, these results indicate that temporary unilateral CCA occlusion alters BBB properties without causing cell death, suggesting a novel model to study the early molecular mechanisms that cause BBB disruption in stroke. This work was supported by The National Council on Science and Technology (CONACYT), Mexico City, Mexico. Grant: CF-2023-G-1516 (MDC).

P1124/B126

Satellite glia to neuron interaction through direct material transfer.

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Glial cells play a critical role in transferring biomolecules to neurons in a limited amount through various pathways, including extracellular vesicles, tunneling nanotubes, synapses, and gap junctions. Using a LoxP-Cre system to label the inner nuclear membrane reporter (Sun1-sfGFP) and ribosomal reporter (Rpl10a-EGFP) in Sox10-Cre and PDGFRa-Cre animals, we discovered that neurons massively receive glial-derived nuclear and ribosomal material, with the functional significance and underlying mechanisms remaining largely unexplored. Satellite oligodendroglia (SOL) in the gray matter are positioned in close proximity to the neuronal soma and have been shown to control neuronal excitability, provide metabolic support to neurons, protect neurons from apoptosis, and can myelinate after demyelinating injury. Notably, we identified SOL-neuron nuclear pairs, correlating with nuclear and ribosomal material transfer. Using transmission electron microscopy we found SOL-neuron pairs with a loss of plasma membrane integrity between nuclei. These findings suggest a novel mechanism of direct intercellular material transfer between SOLs and neurons, expanding our understanding of glia-neuron interactions.

Endosomes and Lysosomes 1: Lysosomal Functions

P1125/B128

Sorting nexin 10 regulates lysosomal enzyme function and ionic homeostasis by controlling PI(3,5)P_2.

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Mutations or ablation of Snx10, a small phosphoinositide-binding sorting nexin, are associated with neurodegeneration, blindness and, notably, osteopetrosis. The similarities between osteoclasts -which malfunction in osteopetrosis- and macrophages, which are developmentally related, prompted us to analyze the role of Snx10 in phagocytosis. Deletion of Snx10 was found to impair phagosome resolution, i.e. the clearance of engulfed targets. This defective resolution was not attributable to failure of the phagosomes to mature (assessed as acquisition of LAMP1) or to acidify. Instead, the inhibition was caused by the reduced Cl^- accumulation within (phago)lysosomes that is necessary for the activation of luminal hydrolases, replicating the phenotype reported in macrophages lacking ClC-7, a lysosomal $2Cl^-/H^+$ antiporter (Wu, J. et al., 2023, *J Cell Biol,* 222(6):e202208155). The delivery of ClC-7 to (phago)lysosomes was unaffected by ablation of Snx10, but the activity of the antiporter was greatly depressed, accounting for the observed Cl- depletion. Snx10 was found to regulate ClC-7 activity indirectly, by controlling the availability of lysosomal PI(3,5)P_2, a rare phosphoinositide that markedly inhibits the activity of ClC-7. By limiting the formation of PI(3,5)P_2, Snx10 enables the accumulation of luminal Cl^- in phagosomes and lysosomes, which is required for their optimal degradative function. Our data suggest that Snx10 regulates the delivery of PI(3)P, the precursor of PI(3,5)P_2, from earlier endocytic compartments to (phago)lysosomes. By controlling the traffic of phosphoinositides, Snx10 regulates the function of late endocytic compartments, accounting for the abnormal phagosomal resolution and possibly also for the failure of osteoclasts to resorb bone that was reported to occur upon mutation or loss of the sorting nexin, resulting in in osteopetrosis.

P1126/B129

A Peroxisome-Derived Lipid and Its Regulation of Lysosomal Function.

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Most lysosomal enzymes use Mannose-6-phosphate (M6P) as a targeting signal to be transported to the lysosome. Deficiency in M6P can cause lysosomal dysfunction, leading to the buildup of undigested material and severe lysosomal storage disorders called Mucolipidosis. We utilized TMEM251 knock-out cells as a Mucolipidosis model, conducting a genome-wide CRISPR-Cas9 knockout screen to identify suppressor mutants that can reverse lysosomal accumulation. Our findings reveal that plasmalogens, lipids synthesized by peroxisomes, have a significant impact on lysosomal function. Knocking out genes related to the plasmalogen synthesis pathway markedly reduces lysosomal accumulation, restores lysosomal function, and clears excess lysosomal contents. Furthermore, we provide evidence that plasmalogen reduction enhances TRPML1-mediated exocytosis, thereby restoring lysosomal function. These findings underscore the important role of plasmalogens in regulating lysosomal function.

P1127/B130

Endolysosome Acidification-Induced Calcium Release Activates Adenylyl Cyclase Increasing Neurite Outgrowth.

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The outgrowth of neuronal neurites is pertinent for new axons, dendrites, and synapses, and it is well-known that calcium (Ca^{2+}) and 3'-5' cyclic adenosine monophosphate (cAMP) play a role in neurite extension. Affected by cAMP, endosomes and lysosomes (hereafter referred to as endolysosomes) contain readily-releasable stores of Ca^{2+}. Therefore, we tested the hypothesis that endolysosome acidification-induced Ca^{2+} release is an upstream event that activates soluble adenylyl cyclase (sAC), increasing cAMP levels and neurite outgrowth. Using SH-SY5Y human neuroblastoma cells, we showed that the endolysosome iron chelator deferoxamine (DFO) and the mucolipin-synthetic agonist 1 (ML-SA1), both (1) acidified endolysosomes, (2) decreased endolysosome Ca^{2+} levels, (3) increased cytosolic Ca^{2+} levels, and (4) increased cytosolic cAMP levels. Increased cAMP activates protein kinase A (PKA) and cAMP increased synapsin I and increased neurite numbers and extension. All of these effects were blocked by the endolysosome-resident TRPML1 Ca^{2+} channel inhibitor mucolipin-synthetic inhibitor 1 (ML-SI1), the specific and allosteric sAC inhibitor LRE-1, as well as the sAC inhibitor SQ22536. These mechanisms highlight the role of drug-induced endolysosome acidification and endolysosome-resident TRPML1 Ca^{2+} channels in neurite outgrowth, suggesting that endolysosome Ca^{2+} release is an early and upstream event in neurite outgrowth. These mechanisms might be targeted to reverse synaptic pruning in neurodegenerative disease states.

P1128/B131

Manipulation of Host Cholesterol by SARS-CoV-2.

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Lysosomes receive extracellular and intracellular cholesterol and redistribute it throughout the cell. Cholesterol egress from lysosomes is critical for cholesterol homeostasis, and its failure underlies the pathogenesis of genetic disorders such as Niemann-Pick C (NPC) disease. We recently reported that the BLOC one-related complex (BORC)-ARL8-homotypic fusion and protein sorting (HOPS) supercomplex is required for the egress of free cholesterol from lysosomes and for the storage of esterified cholesterol in lipid droplets. Depletion of BORC, ARL8, or HOPS does not alter the localization of the lysosomal transmembrane cholesterol transporter NPC1 to degradative compartments but decreases the association of the luminal transporter NPC2 and increases NPC2 secretion. Our further work shows that the supercomplex-mediated NPC2 trafficking is interrupted by the COVID-19 causative virus SARS-CoV-2. SARS-CoV-2 infection causes cholesterol sequestration in the lysosomes, and a screening of SARS-CoV-2 proteins showed that the viral protein ORF3a is primarily responsible. ORF3a directly interacts with the HOPS subunit VPS39. A mutation in ORF3a that blocks this interaction rescues NPC2 lysosomal localization and cholesterol egress. Our study demonstrates that the BORC-ARL8-HOPS supercomplex is required for cholesterol egress from lysosomes and that this machinery is hijacked by SARS-CoV-2, resulting in altered host cholesterol homeostasis.

P1129/B132

SARS-CoV-2 virulence factor ORF3a blocks lysosome function by modulating TBC1D5-dependent Rab7 GTPase cycle.

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SARS-CoV-2, the causative agent of COVID-19, uses the host endolysosomal system for entry, replication, and egress. Previous studies have shown that the SARS-CoV-2 virulence factor ORF3a interacts with the lysosomal tethering factor HOPS complex and blocks HOPS-mediated late endosome and autophagosome fusion with lysosomes. Here, we report that SARS-CoV-2 infection leads to hyperactivation of the late endosomal and lysosomal small GTP-binding protein Rab7, which is dependent on expression of ORF3a. We also observed Rab7 hyperactivation in naturally occurring ORF3a variants encoded by distinct SARS-CoV-2 variants. We found that ORF3a, in complex with Vps39, sequesters the Rab7 GAP TBC1D5 and removes Rab7 from this complex. Thus, ORF3a blocks the GTP hydrolysis cycle of Rab7, which is beneficial for production of viral particles, whereas the Rab7 GDP-locked mutant strongly reduces viral replication. Interestingly, hyperactivation of Rab7 in ORF3a-expressing cells impaired CI-M6PR retrieval from late endosomes to the trans-Golgi network, disrupting the biosynthetic transport of newly synthesized hydrolases to lysosomes. Furthermore, the tethering of the Rab7- and Arl8b-positive compartments was strikingly reduced upon ORF3a expression. As SARS-CoV-2 egress requires Arl8b, these findings suggest that ORF3a-mediated hyperactivation of Rab7 serves a multitude of functions, including blocking endolysosome formation, interrupting the transport of lysosomal hydrolases, and promoting viral egress.

P1130/B133

Maintenance of Amino Acid Homeostasis through Vacuole and Plasma Membrane Communication.

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Organelle deterioration is a common feature of aging and contributes to a number of age-related diseases. Our lab is interested in understanding how organelles are functionally interconnected within cells and uncovering mechanisms by which organelle dysfunction perturbs cellular homeostasis during aging. Our work in this area focuses on the lysosome (or vacuole in yeast), an acidic organelle that functions in protein degradation as well as metabolite storage and signaling. In previous studies, we used budding yeast to interrogate how vacuole dysfunction impacts cell health during aging, with a specific focus on dissecting the nature of its functional link to another metabolic organelle, the mitochondrion. We found that the essential function of vacuoles in supporting mitochondrial respiration is not linked to its well-known function in autophagy, but rather, it was tied to the role of vacuoles in amino acid compartmentation. Specifically, we showed that impaired storage of amino acids in vacuole-inhibited cells disrupted mitochondrial respiration by altering availability of cellular iron. To identify genes and/or pathways that cooperate with vacuoles to maintain amino acid homeostasis and mitochondrial health, we conducted a synthetic lethality screen to uncover processes that become conditionally essential upon vacuole deacidification. Gene ontology analysis revealed that endocytic trafficking (notably components of the ESCRT/MVB pathway) and genes involved in iron homeostasis become conditionally essential upon loss of vacuole acidity. Regulation of plasma membrane (PM) nutrient transporter abundance through the ESCRT pathway is well-characterized, and we found that

vacuole deacidification triggered internalization of PM transporters in an ESCRT- and nitrogen-dependent manner. We also showed that the synthetic lethality between mutations in the ESCRT pathway and vacuole deacidification was rescued by amino acid restriction or iron supplementation, as well as deletion of multiple amino acid transporters. Collectively, our findings reveal an interplay between the vacuole and plasma membrane in maintaining intracellular amino acid homeostasis and suggest that cells adapt to loss of vacuolar amino acid compartmentation by decreasing uptake of amino acids at the plasma membrane.

P1131/B134

Elucidating novel lysosomal regulation of methylated metabolite fluctuations in human health and disease.

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Asymmetric dimethylarginine (ADMA) is a cellular metabolite that becomes elevated in the serum of patients with atherogenesis, hypertension, type II diabetes, and end stage renal disease. While ADMA levels have been shown to correlate with worsening disease outcome, major gaps in knowledge remain in our understanding of the molecular mechanisms of ADMA-mediated signaling in human disease. It is well-established that ADMA is generated from degraded proteins that are modified by arginine methylation. However, there is limited knowledge regarding the proteolytic pathway responsible for ADMA generation. Our preliminary data supports a novel role for lysosomes in ADMA production. My data shows ADMA-modified proteins are delivered to lysosomes in mammalian cultured cells by immunofluorescence confocal microscopy and that genetic depletion of PQLC2, the lysosomal arginine exporter, led to ADMA accumulation in lysosomes. Furthermore, in a lysosomal storage disease model, ADMA similarly accumulated in lysosomes and was correlated with altered signaling pathways that have previously been shown to be responsive to ADMA levels such as nitric oxide synthase (NOS) and its downstream target genes. Moreover, defects in disease cell models could be reversed by exogenous ADMA. My central hypothesis is that ADMA fluctuations are regulated by lysosomes. Further, we propose that elevated levels of ADMA may play a role in the pathogenesis of genetic diseases with altered lysosomal activity. I propose two independent specific aims to address my central hypothesis. My first aim will investigate the role of lysosomes in regulating ADMA fluctuations. We will elucidate whether PQLC2 can export ADMA. Secondly, I will elucidate ADMA's role in altering downstream cellular pathways in a lysosomal storage disease model of cystinosis, which is caused by mutations in a single lysosomal protein. I will test the functional impact of lysosomal ADMA on cell signaling in cystinosis kidney cell models and determine whether exogenous ADMA can rescue downstream defects in target genes and cellular metabolism. At the completion of this work, we will have defined a novel source of ADMA by studying the lysosome. By studying the impact of ADMA in cystinosis, this work will address major gaps in knowledge regarding ADMA regulation and could provide potential therapeutic targets or biomarkers in cystinosis. Given that ADMA levels are high in several human diseases, this line of investigation could provide insight for multiple disease contexts.

P1132/B135

Lysosomes regulate early B cell activation.

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B lymphocytes (B cells) form a vital branch of the adaptive immune system by mounting antibody responses. B cell receptor (BCR)-mediated antigen internalization and further processing for peptide-antigen presentation on MHCII complexes is vital for high-affinity antibody responses and B cell immunological memory. At the same time, B cells serve as antigen presenting cells to activate other immune cells. In our efforts to better understand the regulation of lymphocyte activation, we have focused on characterizing the heterogenous intracellular endolysosomal vesicle pool in B cells, responsible for processing the internalized antigens for peptide-antigen presentation.
To add on the degradative functions of lysosomes, we have found that these vesicles play also a key signaling role upon B cell activation. When the lysosomal functions were challenged by pharmacological targeting using either mildly compromising Cationic amphiphilic drugs (CADs) or lysosome neutralizing Concanamycin A (ConA), B cell activation is severely defected. In more detail, our data point to a key signalling role of lysosomal Ca-channel mucolipin 1 upon antigen exposure. When mucolipin 1 is inhibited, B cells cannot form the activatory contact sites called immune synapses and the BCR-downstream signaling is perturbed, particularly the antigen-triggered intracellular Ca-flux. To build on our studies carried out in B cell lines, we are currently extending our analysis of the signalling function of lysosomes also to mouse and human primary B cells.
In addition to this interesting role of lysosomes in antigen-triggered signalling, we have identified new regulators for the antigen processing capacity of the B cell lysosomal pool, namely the septin cytoskeleton. Using both B cell lines and primary cells from B cell specific Septin7 knock-out mouse model, we have identified a key role for septins in the regulation of lysosomal polarity and antigen processing activity.
Together, our data suggest that unperturbed lysosomal functions are critical in acute B cell activation, also beyond the degradative activities of the lysosomes, and that the septin cytoskeleton regulates the polarity and activity of the lysosomal vesicle pool upon antigen encounter.

P1133/B136

Leucine Aminopeptidase LyLAP enables lysosomal degradation of membrane proteins.

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Transmembrane proteins represent ~20-30% of the human proteome and are subjected to turnover by endocytic uptake and delivery to the lysosome, where their degradation poses a unique challenge due to their hydrophobic, phospholipid-embedded α-helical domains that are inaccessible to endopeptidases. Here, we answer the long-standing question of how lysosomes degrade transmembrane proteins - a key missing piece in the life cycle of this important class of proteins that mediate signal transduction, nutrient import, adhesion, and migration. Combining lysosomal proteomics with functional genomic data mining, untargeted metabolomics, and biochemical reconstitution, we find that a previously mischaracterized enzyme that we rename Lysosomal Leucine Aminopeptidase (LyLAP) is most tightly associated with elevated endocytic activity and enables transmembrane protein degradation. LyLAP performs a unique function not found among known lysosomal hydrolases, namely, processive disassembly of hydrophobic α-helices triggered by their N-terminal hydrophobic (often

Leucine) residues. Importantly, LyLAP is upregulated in pancreatic ductal adenocarcinoma (PDA), an aggressive cancer that relies on macropinocytosis for nutrient uptake. Strikingly, loss of LyLAP activity has catastrophic consequences for lysosomes, ultimately leading to PDA cell death. Thus, LyLAP enables lysosomal degradation of membrane proteins, and may represent a targetable vulnerability in highly endocytic cancer cells.

P1134/B137

Molecular mechanisms for the extracellular digestion of lipoprotein aggregates by macrophages.

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The digestion of aggregated low-density lipoprotein (agLDL) by macrophages and the subsequent accumulation of excessive cholesterol esters in these macrophages in arteries plays a major role in atherosclerotic progression. However, because agLDL is tightly crosslinked to the extracellular matrix, it cannot be digested by conventional endocytosis or phagocytosis mechanisms. Our laboratory discovered a novel mechanism by which macrophages digest agLDL and generate free cholesterol in an extracellular, acidic, hydrolytic compartment known as the lysosomal synapse. Macrophages form a tight seal around agLDL through actin polymerization and deliver lysosomal contents into this space in a process termed digestive exophagy. Vacuolar ATPase on the plasma membrane lowers the pH of the lysosomal synapse, enabling lysosomal acid lipase activity. Lysosomal acid lipase degrades cholesteryl esters in agLDL, generating free cholesterol that accumulates in macrophages, an important hallmark of atherosclerotic plaque.
Our laboratory has begun to characterize the signaling pathways and machinery that regulate digestive exophagy, having identified TLR4 activation of MyD88/Syk as critical for digestive exophagy. Further downstream, Syk activates Bruton's tyrosine kinase (BTK) and phospholipase Cγ2 (PLCγ2). We show that PLCγ2 and to a lesser extent BTK regulate digestive exophagy. PLCγ2 cleaves $PI(4,5)P_2$ into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Soluble IP_3 then activates the release of Ca^{2+} from the endoplasmic reticulum (ER) into the cytoplasm. We demonstrate that Ca^{2+} release from the ER is upregulated by agLDL and plays a role in digestive exophagy. Both DAG and Ca^{2+} activate protein kinase Cα (PKCα). We have also found that PKCα regulates digestive exophagy. Ca^{2+} released from the ER is also critical for refilling lysosomal Ca^{2+} stores. Interestingly, both activation and inhibition of the lysosomal cation channel, TRPML1, inhibit digestive exophagy. TRPML1 has a known role in lysosome exocytosis, so we are currently investigating whether this pathway is required for digestive exophagy. Additionally, to identify novel regulators of digestive exophagy, we are performing a genome-wide CRISPR screen. We hope that expanding our understanding of the mechanisms of digestive exophagy will help identify protein targets for therapeutics to slow atherosclerosis.

P1135/B138

Mapping Lysosome Interaction Landscape by FIB-SEM and Machine Learning Assisted Analysis.

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Lysosomes are central to cellular metabolism and regulation of cellular homeostasis. While primarily known for their catabolic functions such as autophagy, lysosomes also regulate anabolic processes such

as protein synthesis. Many of the lysosome's cellular functions are carried out through the contact of the lysosome membrane with other organelles. Using whole-cell Focused Ion Beam Scanning Electron Microscopy (FIB-SEM), a volume EM method that can reveal the isotropic high-resolution intracellular landscape in cells' native environment, we quantitatively measure lysosomal-related organelles and their contact with the other organelles under various metabolic stresses. We found that inhibition of mTOR, a central nutrient signaling complex, drastically increases lysosome-ER exit site contacts and promotes the degradation of secretory cargos in ER exit sites. We further apply machine learning to quantitatively analyze the lysosome interaction landscape with other organelles in FIB-SEM datasets from cells and brain tissues. We found that lysosome interactions are not limited to membrane-bound organelles, but also include membrane-less organelles like RNA granules. The work revealed lysosomes can interpret metabolic status in cells and reshape the cellular metabolic landscape by globally changing their interaction with other intracellular organelles.

P1136/B139

Lysosome-coupled mRNA transport maintains axonal mitochondrial homeostasis and prevent axonal degeneration.

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Lysosomes were classically defined as cytoplasmic organelles that function to degrade biomacromolecules in the endomembrane system of eukaryotic cells. More recently, lysosomes were found to play multifaceted roles in nutrient sensing, regulation of gene expression, plasma membrane repair, immunity and cholesterol transport. Previous work showed that these functions are influenced by the positioning and motility of lysosomes within the cytoplasm. In particular, we discovered a lysosome-associated, hetero-octameric complex named BORC that sequentially recruits the small GTPase ARL8 and kinesin motors for anterograde movement along microtubules. Interference with this machinery causes redistribution of lysosomes towards the cell center in non-neuronal cells and depletion of lysosomes from the axon in neurons. Based on recent work showing that RNA granules hitchhike on lysosomes for transport into the axon, we examined the effect of knocking out BORC subunits on axonal mRNA transport in human iPSC-derived neurons. We observed that BORC KO caused a dramatic depletion of many mRNAs encoding components of mitochondria and ribosomes. The depleted axonal mRNAs were common with those involved in pathways of neurodegeneration in Parkinson's, Alzheimer's, Huntington's and prion diseases, as well as amyotrophic lateral sclerosis (ALS). A puromycin-proximity ligation assay revealed decreased synthesis of mitochondrial and ribosomal proteins in the axon of BORC-KO neurons. Moreover, immunoblot analyses of BORC-KO axons showed reduced levels of the mitochondrial electron transport chain (ETC) and the mitochondrial contact site and cristae organizing system (MICOS). In addition, we observed that axonal mitochondria were smaller, had disorganized cristae and reduced membrane potential, and were targeted for mitophagy in BORC-KO axons. Finally, we found that BORC-KO axons developed swellings filled with mitochondria, autophagosomes and Tau aggregates, and eventually degenerated. These findings thus demonstrated a critical role of lysosome-coupled mRNA transport into the axon for the maintenance of mitochondrial homeostasis. Failure of this mechanism could explain the pathogenesis of neurodevelopmental disorders caused by mutations in BORC subunits, and, more generally, of neurodegenerative disorders characterized by defective lysosomal transport and mitochondrial function.

P1137/B140

Investigating novel lysosomal regulation of glycolysis for cell growth.

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Skeletal muscle is a highly dynamic tissue with fluctuating metabolic demands. Glycolysis fuels the production of cellular energy in the form of ATP while also providing metabolites to the pentose phosphate pathway (PPP) for redox homeostasis. Phosphofructokinase-1 (PFK1) is the gate-keeper of glucose metabolism and resides at the intersection of glycolysis and PPP metabolic crosstalk. In skeletal muscle, PFKM -which is the muscle specific isoform of PFK1- is responsible for tuning metabolic crosstalk. Loss-of-functional PFKM increases shunting into PPP during the progression of musculoskeletal disease. While intensive efforts have delineated PFKM allosteric inhibitors and activators, major gaps in knowledge remain in the proteolytic pathways that control PFKM turnover, which is an equally important factor in determining metabolic shunting. My preliminary data shows PFKM is rapidly delivered into lysosomes for degradation in cultured cell models. Further, my data shows PFKM is modified by arginine methylation, a post-translational degradative tag for the lysosome, which enables microautophagy. Importantly, PFKM degradation in lysosomes was observed across cell lines derived from multiple tissues including muscle and could be restored by lysosomal inhibitors. Finally, cellular treatments where PFKM was degraded caused shifts in metabolite levels where PPP metabolites were increased. Whether methylation-driven proteolysis regulates PFKM to control skeletal muscle metabolism is unknown. My **hypothesis** is that PFKM is selectively targeted for lysosomal degradation to modulate skeletal muscle metabolism. New therapeutic angles for restoring metabolic balance in musculoskeletal disease could emerge from this work by targeting protein arginine methyltransferases or lysosomal activity.

Mitochondrial Dynamics 1

P1138/B141

Mitochondrial Inner Membrane Remodeling as a Driving Force of Organelle Shaping.

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Mitochondria are dynamic organelles exhibiting diverse shapes. While the variation of shapes, ranging from spheres to elongated tubules, and the transition between them, are clearly seen in many cell types, the molecular mechanisms governing this morphological variability remain poorly understood. Here, we propose a novel shaping mechanism based on the interplay between the inner and outer mitochondrial membranes. Our biophysical model suggests that the difference in surface area, arising from the pulling of the inner membrane into cristae, correlates with mitochondrial elongation. Analysis of live cell super-resolution microscopy data supports this correlation, linking elongated shapes to the extent of cristae in the inner membrane. Knocking down cristae shaping proteins further confirms the impact on mitochondrial shape, demonstrating that defects in cristae formation correlate with

mitochondrial sphericity. Our results suggest that the dynamics of the inner mitochondrial membrane are important not only for simply creating surface area required for respiratory capacity, but go beyond that to affect the whole organelle morphology. This work explores the biophysical foundations of individual mitochondrial shape, suggesting potential links between mitochondrial structure and function. This should be of profound significance, particularly in the context of disrupted cristae shaping proteins and their implications in mitochondrial diseases.

P1139/B142

ATP Synthase Components Distribute in a Stochastic Manner within a Mitochondrial Graph-like Network Structure.

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ATP synthase, pivotal in cellular energy production, is a rotating enzyme anchored in the inner mitochondrial membrane and consists of an F1 and F0 motor. While the structural identification of ATP synthase is well described, the expression, transport, and assembly process have yet to be fully explored. Furthermore, the inconsistent molecular quantity among ATP synthase components highlights the potential formation of sub-complexes and their stoichiometry, such as mitochondrial permeability transition pore (mPTP). We utilized Mitograph software, a fluorescent intensity-based segmentation method, to analyze the distribution of ATP synthase components within the mitochondrial network structure in yeast and evaluated the quantified data using network modeling. Surprisingly, we observed that the probability of the existence of ATP3p, ATP4p, and ATP5p shows distinctive localization at the edge center of mitochondrial tubes, while ATP1p, ATP2p, and ATP7p are evenly distributed throughout the mitochondrial network. In addition, ATP3p and ATP4p accumulated more at the three-way nodes than at the edge center when the network forms three-way nodes. These findings do not support the concept of ATP complex accumulation in the cristae area. Our live-cell microscope observation identified mRNAs encoding ATP synthetase components randomly distributed on the mitochondrial network for co-translationally importing proteins. We then conducted a stochastic computational model to assemble proteins in the mitochondrial network structure. This model of random protein assembly aligned well with the features observed in biological experiments. Our experiments suggested that the components of ATP synthetase may be more diffusively distributed within the mitochondrial network structure and form sub-complexes more often than expected. In addition to developing image processing schemes and modeling methods of protein distribution within graph-like structures, this research accelerated the understanding of mitochondrial ATP synthase protein distribution and assembly process mechanisms.

P1140/B143

Human CCDC51 and yeast Mdm33 are functionally conserved mitochondrial inner membrane proteins that demarcate a subset of fission events.

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Mitochondrial function relies on the ability of the organelle to exist in a dynamic, semi-continuous network that is distributed throughout the cell to meet local metabolic demand. Mitochondrial shape and network organization is largely maintained through opposing fusion and fission dynamics. Fission is mediated by a highly conserved dynamin-related protein (yeast Dnm1/human Drp1), which oligomerizes

around the mitochondrial outer membrane to constrict and divide the organelle through GTP hydrolysis. While extensive work has examined the mechanisms of mitochondrial fission, it remains unclear how fission is coordinated across the two membrane bilayers of the organelle, particularly in metazoans. The yeast inner membrane (IM) protein, Mdm33, has been implicated in facilitating fission, but its role remains controversial because its ablation does not phenocopy the hyperfused mitochondrial network phenotype associated with the loss of other mitochondrial fission factors. Moreover, Mdm33 is not conserved outside of fungal species, calling into question how metazoan cells divide their IM and whether, like in yeast, they also require internal mitochondrial proteins to aid in regulating fission. Here, we use a bioinformatic approach to identify a putative structural ortholog of Mdm33 in humans, the IM protein CCDC51 (also called MITOK). CCDC51 has a similar domain organization to Mdm33, and we find that its stable depletion in mammalian cells leads to mitochondrial morphology defects that mimic those of yeast Δmdm33 cells. Additionally, we find that while CCDC51 is not required for division, transient loss of CCDC51 leads to reduced mitochondrial fission rates and mitochondrial hyperfusion, suggesting that it is involved in Drp1-mediated mitochondrial fission. Consistent with this, we find that overexpression of CCDC51 leads to Drp1 recruitment and promotes Drp1-dependent mitochondrial fission. Furthermore, we spatially and temporally resolve Mdm33 and CCDC51 to a subset of fission events using timelapse microscopy in yeast and mammalian cells, respectively. Finally, we show that exogenously expressed human CCDC51 can partially complement yeast Δmdm33 cells, indicating the proteins are indeed functionally analogous. Our data reveal that human CCDC51 is a novel metazoan ortholog of Mdm33 and suggests that IM proteins play a conserved role in mediating mitochondrial fission.

P1141/B144

Phospholipid Saturation Mediates Remodeling of the Outer Mitochondrial Membrane.

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The lipid composition of membranes underlies organelle function. Membrane phospholipids play roles in the proper localization, conformation, and interactions of membrane proteins. Dysregulation of membrane composition, such as changes in levels of phospholipid species or membrane fluidity, are detrimental to cellular and organismal health. Here, we report that the saturation state of phospholipids mediates remodeling of the outer mitochondrial membrane (OMM). Mitochondrial Derived Compartments (MDCs) are cargo-selective structures that form from the OMM at ER-mitochondrial contact sites. Previous studies have shown that MDCs can remodel the OMM in response to metabolic alterations or protein overload stress in the OMM. We now find that MDCs also form in response to an increase in di-unsaturated phospholipids, where both acyl chains of the phospholipid contain a double bond and create a more fluid membrane environment. Overexpression of *OLE1*, the yeast homolog of Stearoyl-CoA Desaturase-1, increases unsaturated fatty acid synthesis, and promotes MDC formation. In contrast, increasing saturated fatty acid synthesis inhibits MDC formation, suggesting that elevated unsaturated lipids, and not merely an imbalance in lipid saturation, promotes MDC formation. Additionally, unsaturated-lipid induced MDCs are blocked by shunting acyl chains towards lipid droplet formation instead of phospholipid synthesis, suggesting that unsaturated phospholipids ultimately stimulate MDC formation. Recent work in the lab found that disrupting the TOM complex, an import complex on the OMM, leads to MDC formation, and that these MDCs sequester unassembled TOM complex subunits. We found that overexpression of *OLE1* also disrupts the TOM complex and leads to TOM complex subunits localizing to MDCs. Thus, we propose that changes in lipid saturation stimulate

MDC formation by triggering TOM complex disruption in the OMM. Overall, this study uncovers a previously unknown link between phospholipid saturation and TOM complex assembly in the OMM, and uncovers an important role for MDCs in responding to lipid saturation stress.

P1143/B146

Loss of SRP-mediated ER Protein Targeting Induces the Mitochondrial Derived Compartment Pathway.

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Targeting of proteins to specific organelles is a critical function in eukaryotes that is achieved and maintained by a dedicated host of cytosolic protein targeting complexes, organellar import machinery, and quality control machinery. Dysregulation of cellular homeostasis through loss of proper protein targeting is a hallmark of aging and underlies many age-related diseases. Organellar membranes, specifically the outer mitochondrial membrane (OMM), are key interfaces with the cytosol that sense and respond to aberrant proteins and protein import stress. Mitochondrial quality control mechanisms in cooperation with the ubiquitin-proteasome system or autophagy can extract mistargeted/aberrant proteins or remove damaged portions of the organelle. These mechanisms become overwhelmed in the case of potent or prolonged mitochondrial import impairment, and mitochondrially targeted proteins that accumulate in the cytosol are mistargeted to other organelles such as the endoplasmic reticulum (ER), potentially to alleviate proteotoxicity. Inversely, loss of proper targeting to the ER mediated by the signal recognition particle (SRP) results in mistargeting of SRP substrates to the mitochondrial surface. The fate of these substrates - most of which are hydrophobic membrane proteins - and the mitochondrial response have not been interrogated. We previously discovered the mitochondrial derived compartment (MDC), a cargo-selective OMM domain that forms in response to metabolic perturbations and excess protein stress. A recent study proposed a model by which MDCs sequester excess OMM proteins to mitigate proteotoxicity threatening membrane integrity. Here, we performed a microscopy-based conditional knockdown screen of essential *Saccharomyces cerevisiae* genes to identify additional regulators and machinery in the MDC pathway. We found that repression of SRP components, previously shown to result in SRP substrate mistargeting, induced MDC formation. We confirmed this phenotype using an auxin-inducible protein degradation system, demonstrating that induction of the MDC pathway upon depletion of SRP complex components occurred rapidly and was accompanied by severe mitochondrial fragmentation. SRP-induced MDC formation was ablated and mitochondrial morphology was restored when we deleted the mitochondrial protein import receptor Tom70. We hypothesize that mistargeted SRP substrates cause stress at the OMM - potentially through interaction with Tom70 and aberrant insertion into the membrane - and are sequestered into MDCs. Given the MDC's role in sequestering excess mitochondrial proteins, the MDC pathway may have a protective function during SRP-induced protein mistargeting.

P1144/B147

Mitochondrial-Derived Compartments Form a Membrane-Enriched Trap to Sequester Surplus Proteins from the Outer Mitochondrial Membrane.

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Preserving the health of the mitochondrial network is critical to cell viability and longevity. To do so, mitochondria employ several membrane remodeling mechanisms, including the formation of

mitochondrial-derived vesicles (MDVs) and compartments (MDCs) to selectively remove portions of the organelle. In contrast to well-characterized MDVs, the distinguishing features of MDC formation and composition remain unclear. To gain insight into the composition of MDCs, we used electron tomography to observe that MDCs form as large, multilamellar domains that generate concentric spherical compartments emerging from mitochondria at ER-mitochondria contact sites. Time-lapse fluorescence microscopy of MDC biogenesis revealed that mitochondrial membrane extensions repeatedly elongate, coalesce, and invaginate to form these compartments and encase multiple layers of membrane. As such, MDCs strongly sequester portions of the outer mitochondrial membrane (OMM), securing membrane cargo into a protected domain, while also enclosing cytosolic material within the MDC lumen. Remarkably, selective sorting into MDCs also occurs within the OMM, as subunits of the translocase of the outer membrane (TOM) complex are excluded from MDCs unless assembly of the TOM complex is impaired. Considering that overloading the OMM with mitochondrial membrane proteins or mistargeted tail-anchored membrane proteins induces MDCs to form and sequester these proteins, we propose that one functional role of MDCs is to create a membrane-enriched trap that segregates and sequesters excess proteins from the mitochondrial surface. Collectively, our results provide a model for MDC formation, describe key features that distinguish MDCs from other previously identified mitochondrial structures and cargo-sorting domains, and propose a function for MDCs in maintaining the integrity of the OMM.

P1145/B148

Phospholipid conversion in selective mitochondrial fusion.

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Phospholipids are key components of biological membranes, serving as building blocks, influencing membrane structure or acting as signaling hubs. Mitochondria, dynamic organelles regulated by fission and fusion processes, depend on a complex interplay of proteins and phospholipids for their function and quality control. Cardiolipin, a unique phospholipid synthesized and enriched in the inner mitochondrial membrane, promotes mitochondrial division when translocated to the outer mitochondrial membrane. It can be hydrolyzed by phospholipase D 6 (PLD6) into phosphatidic acid, which facilitates mitochondrial fusion. However, the mechanism through which cardiolipin conversion contributes to mitochondrial fusion remain unclear. In this study, we identify the nucleotide diphosphate kinase NME3 as a key player in PLD6-mediated mitochondrial fusion. NME3 is enriched at the contact interface of closely positioned mitochondria in a PLD6-dependent manner and binds directly to PA-exposed lipid packing defects via its N-terminal amphipathic helix. The ability of NME3 to bind PA and form hexamers confer its mitochondrial tethering activity. Notably, nutrient starvation increases the efficiency of NME3 enrichment at mitochondrial contact sites, and the tethering ability of NME3 contributes to fusion efficiency. Our findings establish NME3 as a tethering protein that promotes selective fusion between PLD6-remodeled mitochondria, thereby contributing to mitochondrial quality control.

P1146/B149

Quantifying the Conformational Dynamics of Mfn1 Throughout GTP Hydrolysis.

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The shape, size, and cellular distribution of the mitochondrial network is regulated by dynamic properties including mitochondrial fusion, division, and microtubule-based cell transport. Outer mitochondrial membrane fusion is mediated by the mitofusin paralogs: Mfn1 and Mfn2. Dysregulation of mitochondrial fusion has been implicated in several neurological diseases including Charcot Marie Tooth Type 2A (CMT2A). CMT2A is caused through mutations in *MFN2*, which alter mitochondrial fusion and transport in neurons. The mitofusins are members of the dynamin superfamily, large GTPases that harness GTP-dependent conformational changes to remodel cellular membranes. The mechanism of outer mitochondrial membrane fusion is poorly understood, in part due to a lack of tools to dissect allosteric regulation of mitofusin, which is critical for membrane fusion. Mitofusin proteins physically interact across two mitochondria to initiate fusion and then must dynamically rearrange to pull the membranes together in a way that leads to lipid mixing. We predict that the discrete and progressive steps of membrane fusion are triggered by changes in the nucleotide binding pocket during GTP hydrolysis. Defining this allosteric regulation would provide critical insight into the mechanism of mitofusin-mediated membrane fusion. Additionally, as many CMT2A variants maintain catalytic activity but are defective in membrane fusion, we predict that aberrant allostery is a major component of dysfunction and understanding allosteric regulation of the mitofusins will further elucidate the mechanism of disease. To illuminate the conformational dynamics of Mfn1, we utilized a novel transition metal fluorescence energy transfer system and the minimal catalytic domain. We incorporated an unnatural amino acid as the fluorescence donor and use a cysteine-reactive chelator bound to a divalent cation as the acceptor. This approach allows us to calculate the distance between the donor and acceptor, thus providing structural information. Using fluorescent lifetime measurements, we capture the distribution of mitofusin protein conformations within a population in solution. These population distributions are a measure of the conformational heterogeneity of the protein, which can be altered by ligand binding or disease-associated amino acid substitutions. We determined the conformational state of Mfn1 with different GTP analogs to capture the progressive steps of GTP hydrolysis that allosterically regulate mitofusin-mediate membrane fusion. This powerful system allowed us to capture the GTP-bound state and the unbound mitofusin structure, which have not been reported. Therefore, our data reveal novel insights into the energetics of GTP-driven conformational changes of Mfn1.

P1147/B150

Investigating Septins as Regulators of Mitochondrial Dynamics.

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Mitochondria are highly dynamic organelles that undergo changes in morphology, distribution, and function to adapt to the changing needs of the cell. This remodeling is predominantly carried out by the opposing processes of fission and fusion. Decades of biochemical, biophysical, and imaging studies have demonstrated the requirement of coordinated action of various cytosolic factors including dedicated GTPases, organelles such as ER, and cytoskeletal elements for productive mitochondrial fission. However, how recruitment of this machinery is coordinated at the mitochondrial outer membrane

remains unclear. Untangling the mechanisms involved in mitochondrial membrane scission has proven technically challenging due to the difficulty of visualizing the organization of the fission machinery with diffraction limited imaging techniques, and rapid rates of mitochondrial fission. In previous work we used cryo-electron tomography to interrogate cytoskeletal-membrane interactions at mitochondrial constriction sites. Our work revealed coordination of known fission elements, actin and microtubules, with another cytoskeletal protein, septin. However, the role of septins in coordinating mitochondrial fission has remained unclear. To understand how septins can drive assembly and function of the fission machinery, we use a combination of confocal and super-resolution microscopy. We observe association of septins with mitochondrial membranes in live cells, specifically at sites of membrane constrictions, associated with fission events. To capture the near-native spatial relationship between septins and mitochondria, we employ a vitrification and freeze substitution protocol adapted for stimulated emission depletion (STED) microscopy. We find a pool of the septin cytoskeleton circumventing mitochondrial membrane constriction sites. This association was observed across multiple cell types, supporting a universal role for septin in regulating mitochondrial membrane remodeling. Our work provides a workflow for preservation and visualizing of in-vivo membrane-cytoskeleton interactions and comprehensively demonstratres the first mitochondria-septin interactions under physiological conditions. In future, we aim to delineate the molecular interaction between septins and mitochondrial membranes and the physiological relevance of this interaction.

P1148/B151

Uncovering the role of MSTO1 in mitochondrial fusion and mtDNA maintenance.

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Mitochondria are constantly fusing, dividing, and moving on microtubules to maintain structural and functional integrity. Mitochondrial dynamics are regulated by cytosolic factors and post-translational modifications, providing critical integration into cellular physiology. In addition, maintenance of mtDNA is essential for mitochondrial health, as the genome encodes essential oxidative phosphorylation proteins. Yet, the mechanisms for fusion and mtDNA maintenance are still poorly understood highlighting a large gap in our understanding of mitochondrial dysfunction. Recently, mutations in *MSTO1*, which encodes a cytosolic protein that transiently interacts with the outer mitochondrial membrane (OMM), have been linked to clinical disease phenotypes typical of mitochondrial dysfunction. MSTO1 patient-derived fibroblasts have impaired mitochondrial fusion and a striking loss of mitochondrial DNA (mtDNA). This suggests MSTO1 is a novel regulator of mitochondrial fusion and mtDNA maintenance. Outside of MSTO1 patient studies, nothing is known about the function of MSTO1 in vertebrates, creating a critical gap in our understanding of the molecular mechanisms underlying this disease. MSTO1 protein expression is almost undetectable in MSTO1 patients, indicating that amino acid substitutions destabilize the protein. To recapitulate MSTO1 patient phenotypes, I generated cell lines using the auxin-inducible degradation (AID) system to allow temporal control of MSTO1 protein stability. By four hours of auxin treatment MSTO1-FLAG-AID protein decreases to almost undetectable levels by Western Blot. MSTO1-AID cells treated with auxin for six days possessed shorter mitochondria than controls and MSTO1-AID cells treated with auxin for only three days. These data are consistent with a model where MSTO1 may indirectly regulates mitochondrial fusion due to the extended length of time required for structural changes to occur after MSTO1 is depleted.

P1149/B152

Real-Time Visualization and Analysis of Mitochondrial Fission and Fusion with Holotomography imaging.

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The balance between mitochondrial fission and fusion is essential for cellular homeostasis, with imbalances implicated in numerous diseases, making these dynamics essential biomarkers. However, morphological analysis of mitochondria using fluorescence and electron microscopy is limited by phototoxicity, photobleaching, and an inability to capture live cell dynamics. This study utilizes Holotomography (HT) imaging with the HT-X1 system for non-invasive, real-time, and 3D analysis of mitochondrial morphology without the need for fluorescent staining. We specifically examine mitochondrial structural changes in Hep3B cells following MPP+ and Mdivi-1 treatments, capturing dose-dependent and temporal mitochondrial dynamics. Through HT, we observed significant morphological changes, quantitatively assessed using TomoAnalysis software enhanced with machine learning for accurate mitochondrial segmentation and length measurement. Importantly, utilizing a 96-well format, this methodology allows for simultaneous imaging and analysis of multiple wells under various conditions, significantly enhancing throughput and experimental efficiency. Through this methodology, we achieved detailed mitochondrial segmentation from HT images, enabling the measurement of individual mitochondrial lengths and the quantitative assessment of their distribution changes in response to drug dosage and over time. These sophisticated methodological approaches substantially advance mitochondrial research, providing reliable tools for biomarker identification and a more detailed understanding of the cellular processes involved in disease pathogenesis.

P1150/B153

Perturbing mitochondrial fusion disrupts neuronal form and function*in vivo*.

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Neurons are large cells with complex structures and high energy demands. Mitochondria not only supply energy, but also undergo dynamic changes in morphology through fission and fusion. Imbalance of mitochondrial fission and fusion is associated with synaptic dysfunction and neurodegeneration in cell culture and whole organisms. However, it is unclear how mitochondrial fission and fusion contribute to mitochondrial homeostasis and neuronal form and function in specific cell types *in vivo*. In this work, we used the GAL4/UAS system to selectively perturb mitochondrial fusion in *Drosophila* horizontally system (HS) neurons. We knocked down and overexpressed Marf or Opa1 - which promote fusion of mitochondrial outer or inner membrane, respectively - either throughout development and adulthood or in an adult-restricted fashion. We found that constitutive knockdown (KD) of either Marf or Opa1 leads to mitochondrial fragmentation while overexpression leads to mitochondrial elongation. In Marf KD neurons, we also observed mitochondrial mis-localization, with mitochondria depleted from the dendrite and enriched in the cell body, suggesting that Marf regulates mitochondrial transport as well as mitochondrial fusion. Consistent with this observation, we found that Marf KD reduced anterograde mitochondrial transport through HS primary dendrites. We also found that constitutive Marf KD resulted in reduced neuron count per optic lobe and loss of dendrite complexity. In contrast, adult-restricted Marf KD caused mitochondrial fragmentation and mis-localization but had no effect on the number and

shape of HS neurons, indicating Marf is necessary for the development but not the maintenance of HS form. To investigate the effect of adult-restricted Marf KD on the function of HS neurons, we used *in vivo* 2-photon microscopy to measure visual stimulus-driven Ca^{2+} response in HS dendrites. We found that adult-restricted Marf KD had no effect on Ca^{2+} responses in young flies but significantly reduced Ca^{2+} response amplitudes in old flies. Altogether our results demonstrate that Marf is necessary for the development of neuronal form and the maintenance of neuronal function during aging.

P1151/B154

Differentiation activates mitochondrial OPA1 processing in myoblast cell lines.

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Mitochondrial optic atrophy-1 (OPA1) plays key roles in adapting mitochondrial structure to bioenergetic function. When transmembrane potential across the inner membrane ($\Delta\psi_m$) is intact, long (L-OPA1) isoforms shape the inner membrane through membrane fusion and the formation of cristal junctions. When $\Delta\psi_m$ is lost, however, OPA1 is cleaved to short, inactive S-OPA1 isoforms by the OMA1 metalloprotease, disrupting mitochondrial structure and priming cellular stress responses such as apoptosis. Previously, we demonstrated that L-OPA1 of H9c2 cardiomyoblasts is insensitive to loss of $\Delta\psi_m$ via challenge with the protonophore carbonyl cyanide chlorophenyl hydrazone (CCCP), but that CCCP-induced OPA1 processing is activated upon differentiation in media with low serum supplemented with all-*trans* retinoic acid (ATRA). Here, we show that this developmental induction of OPA1 processing in H9c2 cells is independent of ATRA; moreover, pretreatment of undifferentiated H9c2s with chloramphenicol (CAP), an inhibitor of mitochondrial protein synthesis, recapitulates the $\Delta\psi_m$-sensitive OPA1 processing observed in differentiated H9c2s. L6.C11 and C2C12 myoblast lines display the same developmental and CAP-sensitive induction of OPA1 processing, demonstrating a general mechanism of OPA1 regulation in mammalian myoblast cell settings. Restoration of CCCP-induced OPA1 processing correlates with increased apoptotic sensitivity. Moreover, *OPA1* knockdown indicates that intact OPA1 is necessary for effective myoblast differentiation. Taken together, our results indicate that a novel developmental mechanism acts to regulate OMA1-mediated OPA1 processing in myoblast cell lines, in which differentiation engages mitochondrial stress sensing.

P1152/B155

Elucidating Septin9 in mitochondrial fission.

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Mitochondria continually undergo fission, the process of division, which is indispensable for maintaining the mitochondrial network morphology and health. Mitochondrial fission sites are marked by the endoplasmic reticulum which drives local polymerization of actin to constrict the mitochondrial diameter at the fission site. Septins, a major component of the cytoskeleton, are a family of filament-forming eukaryotic proteins that can both directly and indirectly modulate other cytoskeleton components, including actin. Septins have been implicated in mitochondrial fission, however a connection between septins and regulating cytoskeletal machinery driving the fission process was not identified. We find that SEPTIN9 is critical for mitochondrial fission and enriches at mitochondrial fission

sites with the ER, and prior to the fission factor dynamin related protein 1 (Drp1). SEPTIN9 has an isoform-specific role in fission, dependent on its interaction with a RhoA guanine nucleotide exchange factor, ARHGEF18. Furthermore, locally inducing RhoA activity on mitochondria can restore fission in the absence of SEPTIN9. Since SEPTIN9 is unique to septin octamer complexes, these findings implicate septin octamers in fission and presents an upstream mechanism of tuning the cytoskeleton at fission sites via the actin regulator, RhoA.

P1153/B156

POLDIP2 governs mitochondrial DNA elimination during spermatogenesis to guarantee maternal inheritance in *Drosophila melanogaster*.

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Maternal inheritance of mitochondrial DNA (mtDNA) is highly conserved in metazoans. While many species eliminate paternal mtDNA during late sperm development to foster maternal inheritance, the regulatory mechanisms governing this process remain elusive. Through a forward genetic screen in *Drosophila*, we identified *Poldip2*, a gene predominantly expressed in the testis. Disruption of *Poldip2* led to substantial mtDNA retention in mature sperm and subsequent paternal mtDNA transmission to progeny. Further investigation via imaging, biochemical analyses and ChIP assays revealed that POLDIP2 is a mitochondrial matrix protein capable of binding to mtDNA. We further showed that CLPX, the key component of a major mitochondrial protease, binds to POLDIP2 to co-regulate mtDNA elimination in *Drosophila* spermatids. This study sheds light on the mechanisms underlying mtDNA removal during spermatogenesis and underscores the pivotal role of this process in safeguarding maternal inheritance.

P1154/B157

A nuclease barrier in the inter membrane space prevents Drp1-assocaited mtDNA escape.

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Mitochondria contain a genome (mtDNA) encoding a handful of proteins essential for cellular respiration. Leakage of mtDNA into the cytoplasm can drive a fitness defect. The first genes associated with mtDNA escape were discovered in yeast and aptly named “yeast mitochondrial escape” (Yme) genes. Here, we identify an intermembrane space nuclease, Endonuclease G (human *ENDOG*; yeast *NUC1*) that prevents the escape of mtDNA to the cytoplasm in yeast via its nuclease activity and mitochondrial intermembrane space localization. Strikingly, the loss of yeast Drp1 (Dnm1) prevents the release of mtDNA, implicating a role for mitochondrial fission in mtDNA escape. Finally, we find that the loss of key fission regulators including Gem1, Mdm34, Caf4, Atg44, and Mdm33 promotes mtDNA escape and that deletion of Dnm1 ubiquitously blocks mtDNA escape in these mutants. Overall, our results implicate Drp1-mediated mitochondrial fission as a major mtDNA escape pathway in yeast and that this escape is normally mitigated via the degradation of mtDNA by Endonuclease G.

Nuclear Dynamics 1

P1155/B159

Colony context and size-dependent compensation mechanisms give rise to variations in nuclear growth trajectories.

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Understanding how cellular variations arise across multiple spatial and temporal scales in a population of putatively identical healthy individual cells is fundamental to understanding cell behavior. We focused on nuclei in cultured human induced pluripotent stem cell (hiPS cell) colonies from the Allen cell collection (allencell.org) as a model system to ask this question. We generated a standardized 3D timelapse dataset of thousands of tracked nuclei, visualized via mEGFP-tagged lamin B1 including over 1,000 full-interphase trajectories with several hundred spanning multiple generations. We developed a Vision Transformer-based deep-learning segmentation and quantitative validation workflow to generate high resolution 3D nuclear segmentations. We then tracked these single nuclear segmentations and extracted quantitative features of their size and shape to analyze how nuclear growth varies over multiple timescales and across the multicellular population. We also created reusable open-source tools for image analysis and timelapse data exploration, including an interactive viewer (timelapse.allencell.org) for exploring the multi-scale spatial and temporal variations of any of the quantitative nuclear features derived from this dataset. On the timescale of days, nuclei exhibit changing spatiotemporal patterns in height as colonies grow. On the timescale of the cell cycle we found that individual nuclear volume growth trajectories displayed a range of shapes, from sub- to super-linear. These changes in growth rate arise from shorter timescale variations that could be attributed to their spatial and temporal context within a cell colony and collective cell behavior as nuclei in local neighborhoods grow faster or slower together. In contrast, we identified a strikingly time-invariant population-wide volume compensation behavior whereby individual nuclei tuned their growth duration based on their starting volume to achieve collective size control. Notably, we found that both the starting volumes and the growth durations of nuclei were more correlated for related nuclei than a control set born at a similar time and location. These results indicate that on one hand, inheritance plays a crucial role in determining these two key volume compensation-related growth features. On the other hand, all other analyzed measurements of nuclear growth are determined by when and where the nucleus is within the colony.

P1156/B160

Identifying Mechanisms Required for Nuclear Rejuvenation during Gametogenesis.

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Lifespan is reset during gametogenesis in budding yeast. One pathway that may contribute to lifespan resetting is the exclusion of nuclear pore complexes (NPCs) and age-associated damage from gametes. During meiosis, NPCs and nuclear damage are sequestered into an intracellular compartment called the Gametogenesis Uninherited Nuclear Compartment (GUNC), and ultimately destroyed. However, the mechanisms by which nuclear material is selectively excluded remain unknown. To approach this question, we conducted a genome-wide screen using the yeast deletion collection to identify factors involved in nuclear exclusion during gametogenesis. The screen identified mutants that disrupt the elimination of genetically encoded multimeric nanoparticles (GEMs), which are normally sequestered to the GUNC. In parallel, we are employing a candidate-based approach to determine whether essential genes involved in asymmetric segregation during mitosis are also required for the exclusion of senescence-associated factors during gametogenesis. Identifying genes required for nuclear rejuvenation will provide fundamental insights into the cellular quality control mechanisms that ensure gamete fitness and may illuminate evolutionarily conserved pathways for cellular rejuvenation.

P1157/B161

Investigating Organelle Inheritance and Quality Control Pathways During Gametogenesis.

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Organellar defects accumulate during aging and the progression of age-associated diseases. Recently, we found that the budding yeast *Saccharomyces cerevisiae* can eliminate organellar damage and restore lifespan through meiotic differentiation, namely gametogenesis. During this process, organellar defects such as protein aggregates, extrachromosomal DNA circles, and aberrant nucleolar materials are sequestered in a membrane-bound compartment called the Gametogenesis Uninherited Nuclear Compartment (GUNC), which is degraded after gamete formation. In addition, the ectopic expression of the meiosis-specific transcription factor *NDT80* prevents nucleolar damage and extends lifespan in vegetatively growing cells. These results indicate that meiotic factors that regulate organelle quality control can potentially promote organelle homeostasis and cellular rejuvenation outside of gametogenesis. Despite the importance of these factors, our mechanistic understanding of how organelle quality control is regulated during gametogenesis is limited. To uncover the factors that regulate the elimination of organellar defects during meiosis, we conducted an imaging-based genomic screen on a yeast homozygous deletion collection. From this screen, we identified at least 90 individual gene deletions that may affect the inheritance of nuclear materials during gametogenesis. We are now investigating how these factors eliminate organellar damage at a mechanistic level, as well as determining whether they promote cell rejuvenation. Additionally, we are dissecting the composition and the formation pathway of the GUNC using biochemical methods. Characterizing the organelle quality control mechanisms during gametogenesis will lead to insights into meiotic rejuvenation at the molecular and cellular level, which might reveal pathways that can be leveraged to counteract cellular aging.

P1158/B162

Characterizing Changes to Nuclear Permeability During Budding Yeast Meiosis .

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The nuclear envelope (NE) functions to separate nuclear material from cytoplasm. In the budding yeast *Saccharomyces cerevisiae*, mitotic divisions and the first meiotic division maintain nuclear integrity. However, during meiosis II, we identified a transient change in the nuclear barrier which allows for nucleoplasmic proteins to diffuse throughout the cell. Notably, the NE remains largely intact by electron microscopy (EM). This phenomenon also occurs in the fission yeast *Schizosaccharomyces pombe*, but its regulation and function remain enigmatic in both yeasts. This project aims to investigate these changes in NE permeability in *S. cerevisiae.*

P1159/B163

Mitochondrial-Nuclear Crosstalk During Budding Yeast Gametogenesis.

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Gametogenesis is the conserved process that gives rise to haploid gametes, which are required for sexual reproduction. During gametogenesis in budding yeast, essential cellular components (such as nuclear genomes and mitochondria) are segregated to the forming gametes, while age-associated damage (including abnormal nucleolar material and protein aggregates) is excluded. Exclusion of age-associated damage, as well as core nucleoporins, is driven by sequestration to a fifth nuclear compartment known as the GUNC (Gametogenesis Uninherited Nuclear Compartment) by growing gamete plasma membranes during meiosis II. Concomitantly, extensive membrane contact sites are established between this nuclear compartment and the mitochondrial network. To determine whether this mitochondrial-nuclear contact site plays a role in GUNC formation, I examined loss-of-function mutants for mitochondrial tethers that localize to this meiotic mito-nuclear contact site, including ERMES, *LAM6*, and *CNM1*. These tethers appeared dispensable for GUNC formation, as well as normal meiotic mitochondrial dynamics and gamete development; however, *TOM70*, a mitochondrial import receptor that participates in tethering organelles and other substrates to the mitochondrial surface, was required for proper GUNC formation. In *tom70Δ* mutant cells, nucleoporins are no longer efficiently sequestered to the GUNC.

P1160/B164

Investigating the Role of the LINC Complex in Nuclear Morphology Regulation Using*Xenopus laevis*Egg Extracts.

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Mechanisms controlling nuclear morphology are important in cell biology, especially when it comes to diseases such as cancers and laminopathies. One family of proteins that has been implicated in the regulation of nuclear morphology is the Linker of Nucleoskeleton and Cytoskeleton complex (LINC). The LINC complex consists of two groups of proteins, KASH proteins like Nesprins located in the outer nuclear membrane and SUN proteins localized to the inner nuclear membrane. SUN and KASH proteins

interact in the perinuclear space to span the nuclear membrane. The LINC complex plays important roles in mechanotransduction, cell migration, and the regulation of nuclear shape and size. Much work elucidating LINC complex function has been performed in intact cells and organisms where pleiotropy can obfuscate direct versus indirect effects. For this reason, we have undertaken studies of LINC complex proteins in *Xenopus laevis* egg extracts in which nuclei form de novo in the absence of transcription, translation, cell cycle progression, a cell cortex, and actin dynamics. This provides a simplified system to study the direct effects of LINC complex manipulation on nuclear morphology. Our immunofluorescence assays and western blots confirm the presence of LINC complex proteins in the *Xenopus* egg. We observe peripheral and intranuclear localization of Nesprin-1, SUN-1, and SUN-2 in nuclei assembled in *X. laevis* egg extract. To further investigate the role of the LINC complex, we performed Nesprin-1 neutralizations that resulted in a significant increase in nuclear size. Our future research will focus on utilizing purified proteins to manipulate LINC complex interactions and to investigate the contribution of microtubules.

P1161/B165

Developing a Chemical Genetic Approach to Study VPS4/ESCRT-III Functions in Human Cells.

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In eukaryotic cells chromosomes are separated from the cytoplasm by the nuclear membrane. Failure to maintain this compartmentalization leads to DNA damage and may result in chromosome fragmentation and rearrangements. However, mechanisms underlying the assembly and maintenance of the nuclear membrane are not fully understood. The formation and repair of nuclear envelopes is initiated by the CHMP7 subunit of the ESCRT-III complex, whose assembly is regulated by the AAA (ATPase associated with diverse cellular activities) mechanoenzyme VPS4. Dissecting VPS4 function in human cells has been difficult for at least two reasons. First, human cells express two VPS4 paralogs, VPS4A and B, with redundant functions. Second, the assembly and repair of the nuclear envelope occurs on the minutes and methods to inhibit VPS4 paralogs on matching timescales are needed to dissect these fast dynamics. To address both challenges, we adapted a recently developed chemical genetics approach. Briefly, a Cys-point mutation is engineered into the AAA domain that sensitizes it to a chemical inhibitor named ASPIRe (allele-specific, proximity-induced reactivity-based inhibitor). In unpublished ongoing work, we generated HeLa cells with VPS4A or VPS4B genes deleted and performed shRNA-mediated depletion of each paralog individually and in combination. As anticipated, the combined loss of VPS4A and VPS4B is lethal, but the single knockouts are viable. In the case of VPS4B, the Cys-point mutation need to sensitize the protein to ASPIRe resulted in partial loss of biochemical activity and expression of the mutant allele in cells did not rescue VPS4B loss of function. Therefore, we used structural data to design Cys-point mutations that preserved VPS4 function but retained ASPIRe-sensitivity. Using these VPS4 alleles and ASPIRe-analogs, we probed the function of VPS4 paralogs in human cells. VPS4 inhibition resulted in nuclear membrane-associated CHMP7 polymer accumulation within 4 hours. CHMP4B, the major ESCRT-III protein also associated with these polymers. Control cells, that express the wild-type VPS4 allele did not reveal any of these phenotypes, indicating that these phenotypes are due to loss of VPS4 activity. Together, these data show that we can acutely and selectively probe the role of VPS4 paralogs during the fast dynamics of nuclear membrane assembly and maintenance.

P1162/B166

Dephosphorylation of Nucleoporins by Calcineurin Regulates Nuclear Import and Export Rates.

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Calcineurin(CN), the ubiquitously expressed, Ca^{2+}/calmodulin-regulated proteinphosphatase, is essential for T-cell activation and inhibited by theimmunosuppressants cyclosporin A and tacrolimus (FK506). However, these drugscause a myriad of adverse effects by inhibiting calcineurin in non-immune cells,where many calcineurin targets remain to be identified. This indicates apressing need to understand calcineurin's many roles in humans. Recently,multiple nuclear pore proteins (Nups) including nuclear basket proteins, Nup153and TPR, were identified as calcineurin substrates in vivo and in vitro(Wigington et al., 2020). Nups are components of the nuclear pore complex(NPC), the sole channel for material traveling between the nucleus and cytosol.Crucially, many Nups contain intrinsically disordered phenylalanine-glycine(FG) repeats, which form a selective barrier that regulates nuclear cargotransport through the NPC lumen. Phosphorylation of FG-nups by ExtracellularSignaling Kinase (ERK) inhibits nuclear transport during oxidative stress andviral infection (Crampton et al., 2008; Kosako et al., 2010). Calcineurindephosphorylates some of these ERK-regulated sites, and treating HeLa cellswith calcineurin inhibitors decreased nuclear accumulation of an NLS-containingreporter protein (Wigington et al., 2020). However, whether calcineurinspecifically regulates nuclear import, nuclear export or both remains to bedetermined. Here, we employ light-inducible nuclear transport probes for importand export (LINuS and LEXY, respectively) and live imaging to show thatcalcineurin inhibition decreases both nuclear import and export rates in humanU20S cells. Further, we demonstrate that activating calcineurin via Ca^{2+}signaling increases nuclear export. Modeling suggests that phosphorylation ofFG-Nups decreases interaction between their intrinsically disordered regions,potentially increasing the distance between Nups in the nuclear channel (Mishraet al., 2019). In light of these results, we hypothesize that calcineurinopposes ERK at the NPC to dephosphorylate FG-Nups and increase the density ofthe FG-Nup meshwork, promoting nuclear transport.

P1163/B167

NPCs come in different shapes and sizes - Insights into structural heterogeneity from *in situ* cryo electron tomography.

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The nuclear pore complex (NPC) is a ~100 nm-wide channel that mediates transport across the nuclear envelope. Hundreds of ~30 distinct proteins assemble in a highly modular fashion to form three stacked rings that are rotationally symmetric around the transport channel. The NPC core structure is additionally symmetric across the membrane plane. Distinct sets of peripheral components binding exclusively on the nuclear or cytoplasmic side are essential for transport directionality. How this asymmetry can be maintained despite identical binding interfaces on both sides of the core, remained enigmatic.

We combined subtomogram averaging and 3D template matching with contextual analysis to assess NPCs inside the cell by cryo-electron tomography (cryoET). Studying NPCs outside of the nuclear envelope, we find that the cellular milieu determines NPC composition. While canonical NPCs on the nuclear envelope carry different peripheral substructures on the nuclear and cytoplasmic side, ectopic

NPCs located in membranes within the nucleoplasm or cytoplasm are symmetric across the membrane plane: Cytoplasmic NPCs exhibit substructures typical to the cytoplasmic side on both sides, whereas nucleoplasm-specific peripheral components are bound to both sides of nucleoplasmic NPCs. The symmetric core that is common to all NPCs is more constricted for the ectopic NPCs. Employing live-cell imaging, we demonstrate that the nucleotide state of the small GTPase Ran, a key component of the nuclear transport system itself, determines NPC asymmetry.
In addition to NPCs in different locations, we identify partial NPCs that could represent assembly intermediates: Some recapitulate previously described assembly pathways, which can now be described in a near-native state at high resolution. Others suggest a surprisingly close interplay between membrane fusion and NPC assembly. Additionally, we find NPCs where the rotational symmetry of the stacked rings does not match each other, exemplifying that the NPC structure can accommodate a surprising variability.
Out results illustrate that subcellular location can be a key determinant of protein complex composition and highlight that *in situ* cryoET allows us to describe the heterogeneity inherent to life.

P1164/B168

Identifying the Function of Nuclear Membrane Protein Emerin in Human Skeletal Muscle Differentiation.

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The inner nuclear membrane (INM) protein emerin has been shown to play a role in cardiac and skeletal muscle development and maintenance. Mutations in the protein lead to X-linked Emery-Dreifuss Muscular Dystrophy, a degenerative disease characterized by both muscle wasting and cardiomyopathy. Skeletal muscle development requires a specific cascade of differentiation factor expression for proper embryonic development as well as for postnatal muscle repair. Previous work in primary mouse myoblasts suggested that emerin played a role in the transition from cycling myoblasts to terminally differentiated myotubes via the Rb1/E2F and MyoD pathways, respectively[1]. However, emerin-null mice do not develop muscular dystrophy due to functional compensation by the INM protein LAP1, which is more highly expressed in murine muscle than in human muscle[2]. Murine systems are thus a poor model for dissecting emerin's functions in muscle. To gain insight into emerin's functions in human cardiac and skeletal muscle, we need a tractable human model system. We have established an induced skeletal muscle (iSM) differentiation system to efficiently direct skeletal muscle differentiation from human induced pluripotent stem cells (hiPSCs) by inducible expression of the transcription factor MyoD and the chromatin remodeler Baf60c[3]. Using hiPSC-derived iSMs, we confirm that loss of emerin impairs differentiation of human iSMs into multi-nucleated myotubes that express the skeletal muscle marker myosin heavy chain 1 (MyHC). Additionally, we show that emerin null iSMs contain a significantly higher proportion of MyoD-positive, MyHC-negative nuclei than wild-type iSMs. Emerin null nuclei also fail to assume the proper differentiated morphology, remaining significantly more round than wild-type nuclei. Rescue experiments with wild type and mutant emerin are ongoing. We are also currently determining the ability of these iSMs to exit the cell cycle and transit through differentiation stages by immunostaining for relevant markers. These data suggest that emerin null hiPSC-derived myoblasts are impaired in their ability to respond to pro-differentiation signals to form mature myotubes.
1. Melcon et al. Human Mol Gen 2006 (PMID 16403804) 2. Shin et al. Dev Cell 2013 (PMID 24055652) 3. Albini et al. Cell Reports 2013 (PMID 16403804)

P1165/B169

ESCRT-III-dependent repairs of the nuclear membrane counteract Lamin A/C-associated cardiomyopathy.

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Lamin A/C (*LMNA*) is a nuclear lamina protein that provides structural support to the nuclear membrane. Loss-of-function mutations in *LMNA* cause severe adult-onset dilated cardiomyopathy (LMNA-DCM) that lacks effective treatment except for heart transplantation. We and others recently demonstrated that localized ruptures of the nuclear membrane in cardiomyocytes are the likely pathogenic cause of this disease. Here, we report that the BANF1-ESCRT-III pathway, which drives nuclear membrane reformation during mitosis, repairs ruptured nuclear membranes and counteracts dilated cardiomyopathy in a mouse model of LMNA-DCM. Conditional deletion of *Lmna* in cardiomyocytes of adult mice (*LmnaCKO*) caused frequent localized nuclear membrane ruptures within cardiomyocytes. We found that BANF1-ESCRT-III pathway proteins, including BANF1, LEMD2, CHMP7, CHMP4B, and VPS4B, localized to the surface of chromatin protruding from the rupture sites in *LmnaCKO* cardiomyocytes *in vivo*, suggesting active nuclear membrane repair. Indeed, the ER membrane lumen protein BiP surrounded the protruding chromatin in a subset of ruptured nuclei, indicating restored nuclear membranes. Despite the repair activity, *LmnaCKO* mice died due to cardiomyopathy 4 weeks after *Lmna* deletion. To examine whether the nuclear membrane repair activity had functional significance, we overexpressed dominant-negative VPS4 (VPS4-E235Q) that inhibits VPS4's ATPase activity required for ESCRT-III-dependent membrane repairs. Strikingly, cardiomyocyte-specific VPS4-E235Q overexpression accelerated the development of cardiomyopathy in *LmnaCKO* mice by 1 week, while this overexpression had no adverse effect in wild-type mice. These results suggest that the endogenous BANF1-ESCRT-III nuclear membrane remodeling pathway counteracts LMNA-DCM. The study also highlights that the endogenous repair activity is insufficient to prevent the ultimate disease progression. Thus, enhancing the BANF-ESCRT-III activity is a potential therapeutic approach for LMNA-DCM.

P1166/B170

Investigating the role of mechanical cues in activating nuclear envelope repair in closed mitosis.

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Mitotic division is an essential dynamic process that requires coordination between the chromosomes, the spindle, and the nuclear envelope. Impaired division due to chromosome mis-segregation is a hallmark of cancer, developmental/birth defects, and miscarriages. *S. pombe* fission yeast is a genetically tractable system and serves as robust model organism to study closed mitosis (in which chromosomes segregate without breakdown of the nuclear envelope). The envelope must physically expand, deform, and actively repair itself to remain structurally intact during division, whilst the mitotic spindle must also accommodate mechanical constraints as it elongates in opposition to inward-directed forces from the nuclear envelope. However, how the envelope and spindle coordinate together to dynamically and efficiently maintain force equilibrium is unclear. To address this, we used a laser ablation approach to perturb the nuclear envelope of *S. pombe* yeast. We ablated yeast that express a GFP nuclear localization signal (GFP-NLS), which serves as a readout of nuclear integrity, and monitored the frequency and occurrence of envelope repair at interphase and mitosis. Interestingly, we find that

mitotic nuclear envelopes can repair post ablation, and they do so much more reliably than interphase ones. To delineate differences between repair mechanism at interphase and mitosis, and to elucidate what primes the envelope for repair, we are investigating how the mechanical environment of the nucleus may influence this process. We hypothesize that as the cell transitions from interphase to mitosis and the spindle elongates, the nuclear envelope experiences a shift in its mechanical environment that serves as a cue for potential molecular sensors to signal for the activation of repair machinery. To assess the contribution of envelope mechanics on repair and to identify what molecular players contribute to the process, we are using a combinatorial approach of genetic, pharmacological, and physical perturbations that modify and assess the mechanical environment of the nuclear envelope at interphase and mitosis. Together, our findings will elucidate the mechanism of nuclear envelope repair and its relationship to spindle mechanics and provide valuable insight on how their interactions direct dynamic shape changes during closed mitosis.

P1167/B171

Investigating nuclear envelope budding: a noncanonical mechanism to export large cargoes.

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For decades, the nuclear pore complex (NPC) has been considered the sole route to transport molecules across the NE. However, in recent years, NE budding (NEB) has emerged as an alternative non-canonical route for nuclear export of viral cargoes that are too large to pass through the NPC. The significance of this unconventional export pathway for the export of endogenous cargoes in mammalian cells remains largely unexplored. We hypothesized that NEB could serve as an alternative export mechanism for uniquely long transcripts. The longest transcript found in mammalian cells is titin, which encodes the third most abundant protein found in muscle cells. Here, we used a combination of electron and fluorescence microscopy to demonstrate that NEB events occur coincidently with the differentiation of myoblasts into myotubes and concomitant with the expression of Titin and other long muscle-specific transcripts. We show that NE buds are derived from the inner nuclear membrane, contain internal vesicles, and use single-molecule fluorescence in situ hybridization (smFISH) experiments to reveal they are specifically enriched with uniquely long transcripts. We developed a protein proximity biotinylation strategy to then identify factors enriched on the buds to gain insight into the underlying mechanism of mRNA targeting to NE buds. We demonstrate the role for an RNA binding protein and a membrane remodeling machinery in NEB targeting and NE bud biogenesis. Together, our data demonstrate with mechanistic insight a noncanonical pathway for large transcript packaging and export in muscles cells. Future experiments will probe whether NEB is a noncanonical export pathway for large cargoes in other postmitotic mammalian cell types.

P1168/B172

Nuclear Membrane-Nucleolus Contact Regulates Ribosome Biogenesis.

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Severe nuclear membrane invagination (NMI) is a hallmark of cancers, aging, neurodegeneration, and infections. However, the outcomes of NMI in cell functions remain unclear. This work identified a critical function of NMI: regulating ribosome biogenesis via contacting nucleolus. With expansion microscopy and live cell imaging, we observed frequent physical contact between the nuclear membrane and

nucleoli. Surprisingly, the higher the nuclear membrane curvature, the more ribosomal RNA and pre-ribosomes are made in the contacted nucleolus. By growing cells on nanopillars that generate NMI with desired curvatures, we quantitatively increased and decreased ribosome biogenesis. The nanopillar experiment indicates that the curvature of NMI at the contact site with the nucleolus can regulate the level of ribosome biogenesis. Based on this causation, we repressed the ribosome levels in breast cancer and progeria cells to a normal level by growing cells on low-curvature nanopillars, indicating that overactivated ribosome biogenesis can be rescued by reshaping NMI-nucleolus contact. Mechanistically, high curvature increases RNA polymerase I, reduces heterochromatin, and enriches nuclear pore complexes at the NMI-nucleolus contact, which collectively promote ribosome biogenesis. We anticipate that our findings will serve as a start for further studies on membraneless-membrane organelle interactions.

P1169/B173

Calcium waves and nuclear envelope tension orchestrate epithelial response to mechanical stress.

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Epithelia are continuously exposed to a range of biomechemical forces such as compression, stretch and shear stress arising from their dynamic microenvironments and associated to their function. Changes in tension such as stretch are known to trigger cell rearrangements and divisions and impact cellular transcription until mechanical stress is dissipated. How cells process, adapt and respond to mechanical stress is being intensively investigated. Our previous work showed that creating a single fold within epithelial monolayers by imposing a deformation of an underlying substrate had surprisingly no impact on the transcription of the cells being under tension but was associated with the calcium waves that propagated from the fold outwards. To understand this inconsistency with the present literature we uncovered the role of the calcium waves in the adaptation response of epithelial cells to mechanical stress by combining the innovative method for fold generation, live imaging, mechanobiology tools and chemical screening. Indeed, we observed that in the timescale of minutes nuclei initially deformed upon folding recovered their shape but solely in the presence of calcium. Moreover, we showed that formed folds are associated with the increase in the nuclear envelope tension. By creating a mutant overexpressing LBR that relaxed nuclear envelope, we demonstrated that despite presence of calcium waves, nuclear tension increase was essential to trigger nuclear shape recovery post folding through the activation of cellular contractility in the cPLA2 dependent manner. Overall our results identify molecular mechanism for nuclear shape recovery and indicate that mechanical stress dissipation program is activated at the level of nuclei which serve as internal tension sensors.

P1170/B174

Nuclear Envelope Protein Emerin is a Key Regulator of Tumorigenic Characteristics in Invasive Triple-Negative Breast Cancer Cells and Noninvasive MCF-7 Cells.

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Emerin is an inner nuclear membrane protein that maintains nuclear structure and rigidity by binding to nucleoskeletal partners at the nuclear envelope. In breast cancer cells, the size and rigidity of the

nucleus is inversely correlated with metastasis. Our lab showed triple-negative breast cancer (TNBC) cells had 50% less emerin protein and smaller, dysmorphic nuclei, resulting in increased cell invasion and tumor formation compared to normal breast cells. When TNBC cells expressing exogenous emerin were injected into a murine breast cancer model, both primary tumor volume and metastasis were significantly decreased compared to vector-only controls. Thus, we concluded that emerin was a tumor suppressor. We predicted that reducing emerin protein expression in noninvasive cancer cells would drive an invasive phenotype, marked by decreased nuclear area and volume, increased cell migration, and increased cell proliferation. To test our hypothesis, we created stable emerin shRNA knockdown lines in noninvasive MCF7 cells and invasive MDA-231 cells and measured nuclear area and volume, cell migration, and cell proliferation. Emerin reduction by 80% in noninvasive MCF7 cells resulted in a more invasive phenotype. This reduced emerin protein expression caused MCF7 nuclei to become smaller and more malleable, resulting in increased impeded cell migration. Since emerin reduction drives cancer invasion, and emerin expression rescues tumorigenic effects of TNBC cells, which have 50% less emerin, we predict increasing emerin expression will block tumor progression and metastasis in patients. Thus, we tested an approach to deliver emerin mRNA into TNBC cells using lipid nanoparticles (LNPs) due to their low toxicity, as demonstrated by the COVID-19 vaccine. We found that emerin mRNA can be successfully delivered to MDA-231 and MCF7 cells, increasing emerin protein expression. We will continue optimizing LNP delivery to minimize toxicity while maximizing protein expression and stability. The ability of emerin LNPs to rescue tumorigenic effects seen in TNBC cells will also be tested. Collectively, these studies demonstrate that emerin is a key regulator of tumorigenesis and metastasis by acting as a tumor suppressor that will be targeted with emerin LNP delivery to rescue tumor growth and metastasis.

Endoplasmic Reticulum and Golgi Structure, Function, and Vesicular Transport 1

P1171/B175

The Endoplasmic Reticulum as an Active Liquid Network.

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The peripheral endoplasmic reticulum (ER) forms a continuous, dynamic network of membrane-bound tubules in eukaryotic cells. While individual structural elements and the morphogens that stabilize them have been described, a comprehensive understanding of the dynamic large-scale network topology remains elusive. We develop a physical model of the ER as an active liquid network, governed by a balance of tension-driven shrinking and new tubule growth. This simplified model, which exhibits connections to prior work on epithelial sheets and crystal grains, results in steady-state network structures with density and rearrangement timescales predicted from the junction mobility and tubule spawning rate. Various parameter-independent geometric features of the liquid network model are shown to match the ER architecture in live mammalian cells, with no fitting parameters. The liquid network model links the timescales of dynamic features such as ring closure and new tubule growth in

the ER. Furthermore, it illustrates how the steady-state network morphology at the cellular level emerges from the balance of microscopic dynamic rearrangements.

P1172/B176

Mechanical Compression Accelerates ER Luminal Diffusivity.

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The extracellular mechanical environment regulates cell behavior during tissue development and homeostasis. Recent studies have illuminated the influence of mechanical forces on the interactions between the endoplasmic reticulum (ER) and various cellular constituents. However, whether and how the ER lumen senses and reacts to mechanical stimulation remains elusive. Here, we utilized ER-targeted Genetically Encoded Multimeric nanoparticles (ER-GEMs) to probe ER luminal diffusivity by single particle tracking. Our findings reveal that mechanical compression notably enhances the diffusivity of ER-GEMs, marking a distinct response when compared to the mechanical behavior of the cytosol. Subsequently, we demonstrated that this increase in diffusivity is not attributable to osmotic compression or the unfolded protein response. Further investigation indicated that the augmented luminal diffusivity could be rescued by depleting calcium or ATP levels, and by reducing the contractility of myosin II. Finally, we have shown that the nucleus, which has been identified as a mechanical sensor under confinement, can transduce compression to ER and accelerate ER luminal diffusivity. Future studies will aim to elucidate the role of luminal diffusivity in modulating secretion and facilitating cellular adaptation to mechanical stimuli. <!--EndFragment-->

P1173/B177

Identification of novel factors activating ATL2-mediated ER membrane fusion.

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The intricate mesh-like structure of endoplasmic reticulum (ER) is generated by homotypic membrane fusion between ER tubules, mediated by the evolutionarily conserved dynamin-like GTPase, atlastin. In previous studies, we showed that all human atlastins are sufficient to induce fusion when reconstituted into liposomes with a lipid composition mimicking that of the ER, suggesting that atlastin is the major fusogen for ER membrane fusion. Intriguingly, however, we observed efficient fusion between ER microsomes isolated from cultured, non-neuronal cells that predominantly express ATL2-1, an autoinhibited isoform of ATL2, ATL2-1 failed to support liposome fusion by itself as reported previously, indicating that cellular factors enable ATL2-1 to mediate ER fusion in vivo. Consistent with this idea, the isolated cytosol from HEK293 cells can induce ATL2-1 mediated liposome fusion. Here, we introduce Affinity Purification Mass Spectrometry (AP-MS) to identify cytosolic factors that regulate the function of ATL2 through its interactions. We identify that a Rab GTPase, previously reported to regulate ER structure, physically interacts with ATL2. Fusion between ER microsomes isolated from HEK293 cells, where ATL2 is the major atlastin, is inhibited by anti-Rab GTPase antibodies. Furthermore, the co-reconstitution of a Rab GTPase into liposomes markedly enhances the fusion of ATL2-containing liposomes. Our findings suggest that a Rab GTPase promotes ATL2-mediated ER fusion and provide insights into the proteome that interacts with ATL2 in the cytosol.

P1174/B178

The ER serves as a microtubule organizing center to promote organized microtubule disassembly during Compartmentalized Cell Elimination.

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Cell elimination is a key feature of both development and homeostasis, as well as pathological conditions. Specialized cells, such as neurons, are defined by elaborate processes bearing different compartments with vastly district subcellular architectures. Morphologically complex cells can undergo regressive events, such as region-specific pruning in neurons. However, the molecular mechanisms behind specialized cell regression remains to be fully understood, with many outstanding questions; for example, how do subcellular organelles in the different compartments contribute to complex cell elimination? We addressed this in the powerful genetic model organism *C. elegans* by studying a novel embryonic cell death program we have previously characterized, Compartmentalized Cell Elimination (CCE). We have observed that CCE occurs in a stereotyped fashion in two sets of polarized cells, an epithelial cell and a set of sex-specific sensory neurons. In these cells, we observe two distinct elimination morphologies in the single process/neurite: proximal beading followed by fragmentation, and distal thickening and shortening (retraction). At the junction of these process/neurite segments, a distal node is formed as cell death progresses. Here we report that CCE involves highly ordered and organized microtubule (MT) dynamics across each process compartment that are disrupted when endoplasmic reticulum (ER) network stability is compromised. We also find stereotyped, and restricted, spatiotemporal ER dynamics, with the ER excluded posterior to the distal node. Our genetics implicates the conserved ER-shaping proteins ATLN-1/Atlastin and LNP-1/Lunapark in MT severing via the conserved ATPase SPAS-1/Spastin. The human variants of all three proteins are implicated in neurodegenerative disease. Interestingly, SPAS-1/Spastin, unlike the ER, is distributed both proximally and distally, suggesting ER-associated and ER-independent dual roles in CCE. Moreover, we find that mutants for the gene encoding PTRN-1/CAMSAP exhibit abnormal CCE. PTRN-1/CAMSAP is a known component of microtubule organizing centers (MTOCs) that functions in MT nucleation. Electron micrographs and two MT reporters show MT accumulation in the distal node. Additionally, two established MTOC markers show signal upon distal node formation. These data lead us to propose that the distal node ER may be a previously undescribed non-centrosomal microtubule organizing center (ncMTOC), playing a role in specialized cell elimination. Ongoing experiments aim to examine this further as well as to explore the dual functions of SPAS-1/Spastin and the spatially-restricted distribution of the ER in CCE.

P1175/B179

Role of the Keratin-Endoplasmic Reticulum Contact Site in Skin Disease.

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Keratins are epithelial-specific intermediate filament proteins encoded by multiple genes that provide mechanical resistance to environmental stressors in tissues such as the epidermis. Epidermolysis bullosa simplex (EBS), a skin blistering disease, is caused by mutations in keratins 5 and 14 (KRT5/ KRT14). Prior studies have reported activation of endoplasmic reticulum (ER) stress and inflammatory pathways in EBS cell culture models. However, the molecular mechanisms linking keratin dysfunction to activated stress

responses in EBS remain poorly understood. We first wanted to determine the association between the ER and desmosome-keratin complex using electron microscopy and live-cell fluorescence imaging in A431 epithelial cells. We found that peripheral ER tubules (labelled with mApple-VAPB) are in close proximity to keratin filaments (labelled with mNeonGreen-KRT14) and form mirror image-like arrangements at desmosome cell-cell junctions. Focused ion beam scanning electron microscopy (FIB-SEM) and 3-D reconstructions reveal intricate nanoscale associations of ER tubules with keratin filaments and the desmosome inner dense plaque. Keratin intermediate filaments align and intertwine with ER tubules at points of contact we term the keratin-ER contact site (KERCS). In addition, analysis of living A431 cells co-expressing mNeonGreen-KRT14 and mApple-VAPB demonstrates that keratin filaments stabilize ER membrane. Disruption of keratin filaments by expression of an EBS-causing keratin 14 aggregation mutant, KRT14^{R125C}, leads to changes in ER morphology, converting ER tubules at the cell periphery to ER sheets. Lastly, FIB-SEM datasets revealed that keratin filaments are present within nanometers of ER-mitochondria contact sites. ER exit sites, which form at high-curvature domains of the ER and are a part of the ER secretory pathway, are also proximal to keratin filaments. To determine the effects of keratin disruption on mitochondrial organization, we labelled mitochondria in cells co-expressing the KRT14^{R125C} aggregation construct and mApple-VAPB. We observed that mitochondria are localized more peripherally in KRT14^{R125C}-expressing cells. Interestingly, mitochondria preferentially associate with the peripheral ER sheets formed by KRT14^{R125C} aggregates, suggesting that ER morphological domains govern the positioning of mitochondria within the cell. Our results demonstrate that keratin filaments regulate the stability and organization of the ER network in epithelial cells. Further, these studies suggest that keratin disruption in diseases alters the organization of other cellular organelles via its effects on ER morphology, potentially leading to organelle dysfunction.

P1176/B180

Investigating the Mechanism for Human Atlastin-2 Autoinhibition.

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The endoplasmic reticulum (ER) is a highly dynamic organelle comprised of sheets and tubules. Tubules fuse together generating three-way junctions, requiring catalysis by the atlastin (ATL) GTPases for membrane fusion. Humans possess three ATL paralogs (1-3) that are both largely conserved and differentially expressed, with ATL2/3 being the predominant paralogs of tissues outside of the central nervous system. Recently, it was discovered that ATL1/2 are both autoinhibited by a short non-conserved C-terminal stretch occurring immediately after their amphipathic helices despite no apparent similarities in this portion of the C-terminus. Significantly, although both proteins are autoinhibited, the degree of inhibition is different with ATL2 being potently inhibited while ATL1 is only partially inhibited. While it is has been demonstrated that removal of this portion called the C-terminal autoinhibitory domain (CAD) activates both paralogs, little is known regarding the mechanism underlying this autoinhibition.

Here I investigate our lab's speculative model for autoinhibition where the CAD constrains ATL's cytoplasmic GTPase/three-helix bundle domains preventing the protein from engaging in the ATL fusion cycle. Through mutagenesis and in vitro reconstitution of fusion activity using purified ATL I probe the mechanism of ATL2 autoinhibition. Results support this model, showing that autoinhibition extends broadly throughout the CAD, with diverse mutations activating autoinhibited ATL2. This work furthers our understanding of how the ATL2 CAD acts to so strongly inhibit fusion, laying the groundwork for

pursuing other outstanding questions in the field such as how autoinhibition is relieved and when relief occurs.

P1177/B181

Curvature dependent morphological reorganization of the endoplasmic reticulum determines the mode of epithelial migration.

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From single-cell extrusion to centimeter-sized wounds, epithelial gaps of various sizes and geometries appear in all organisms. For gap closure, epithelial cells invoke two orthogonal modes: lamellipodial crawling at the convex edge and purse-string-like movements at the concave edge. The mechanisms underlying these differential responses to geometric cues remain elusive. We have performed an intracellular cartography to reveal that in both micropatterned and naturally arising gaps, the endoplasmic reticulum (ER) undergoes edge curvature-dependent morphological reorganizations with convex and concave edges promoting ER tubules and sheets, respectively. This reorganization depends on cytoskeleton-generated protrusive and contractile forces. Additionally, theoretical modeling predicts that the curvature-specific ER morphology leads to a lower strain energy state. ER tubules at the convex edge favor perpendicularly oriented focal adhesions, supporting lamellipodial crawling while ER sheets at the concave edge favor parallelly oriented focal adhesions, supporting purse-string-like movements. Altogether, ER emerges as a central player in cellular mechanotransduction, which orchestrates two orthogonal modes of cell migration by integrating signals from cytoskeletal networks.

P1178/B182

Endoplasmic Reticulum-shaping Proteins of *Plasmodium* are Essential for its Pathology-causing Intraerythrocytic Stage Development.

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Malaria, caused by protozoan parasites of *Plasmodium* species, kills over half a million people annually. Repeated rounds of *Plasmodium* infection and replication in erythrocytes cause disease and if untreated, death. To counter resistance in parasites to frontline treatments, drugs with novel targets and mechanisms of action are urgently needed. The endoplasmic reticulum (ER) of the parasite, the location of several virulence-related features, provides promising opportunities for drug development. The ER consists of morphologically distinct regions including the nuclear envelope, ribosomes-studded sheets and a tubular network. ER sheets are major sites of protein synthesis and transport, while ER tubules are the sites for lipid synthesis, calcium storage, and contacts with plasma membrane and mitochondria. The ratio of these structural elements varies depending on cell type and functional status. *Plasmodium*'s ER morphology is dynamic but there is little information on how it is specified and maintained. In higher eukaryotes, ER tubules are generated and stabilized by integral membrane proteins, YOP1/REEPs and Reticulons (RTNs), which induce membrane curvature. ER tubules are fused together by membrane-embedded dynamin-type GTPases, SEY1 or atlastins. We found *Plasmodium* encodes YOP1, YOP1-like (YOP1L), RTN1 and SEY1. In the rodent-infective parasite, *P. berghei*, YOP1 localizes to the parasite ER and can induce membrane curvature *in vitro*. ΔPbYOP1 parasites display

dysmorphic ER, enlarged digestive vacuoles (DV) and attenuated experimental cerebral malaria. Here, we show that hemoglobin degradation is disrupted in ΔPbYOP1 parasites, suggesting this as a cause for the observed DV enlargement. In the human-infective parasite, *P. falciparum*, knockdown of YOP1 significantly slows parasite growth in erythrocytes. We also demonstrate that depletion of PfSEY1 disrupts schizogony and causes parasite death. In contrast, deletion of either PfYOP1L or PfRTN1 has no impact, suggesting these proteins are either dispensable during the intraerythrocytic cycle. In conclusion, we demonstrate species conservation of YOP1's key role in the pathology-causing intraerythrocytic stages, and provide biological validation for PfSEY1 as a novel drug target for malaria therapy.

P1179/B183

The Actin-Binding Protein Profilin 1 is Important for Maintaining the Integrity of the Endoplasmic Reticulum.

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Profilin 1 (PFN1) is a ubiquitously expressed and crucial actin-binding protein regulating the polymerization of actin from monomers into filaments. Novel roles for PFN1 have been recently identified in the regulation of stress granules, nucleocytoplasmic transport, mitochondria, and microtubules. However, a connection between PFN1 and the endoplasmic reticulum (ER) has yet to be explored. The ER forms a vast, interconnected network essential for protein and lipid synthesis, calcium regulation, and the orchestration of the unfolded protein response. The ER's function is dependent on its morphology as different specialized structures exert varying functions. To determine the relationship between PFN1 and the ER, we used super-resolution microscopy, serial section electron microscopy, and automated segmentation and morphology analysis. We found that PFN1 knockout cells exhibit significant alterations in the ER network, such as changes in ER sheet and tubule ratios, as well as tubule connectivity and fragmentation. Furthermore, serial section electron microscopy similarly showed ER morphology deficits. Functional PFN1-dependent changes were also identified. Additional experiments have indicated that PFN1's role in maintaining ER morphology is not related to its role of polymerizing actin in the cytoplasm. These results reveal a previously unrecognized role for PFN1.

P1180/B184

Investigating the role of the rhomboid protease RHBDL4 in Pancreatic Ductal Adenocarcinoma.

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Pancreatic cancer is a deadly cancer with a survival rate 5 years post-diagnosis of only 10% due to a lack of effective treatments. The predominant form of Pancreatic cancer, Pancreatic Ductal Adenocarcinoma (PDAC), accounts for approximately 95% of all pancreatic cancers. Recent studies demonstrate that the endoplasmic reticulum (ER), an essential organelle where many proteins are folded, is important for promoting cancer growth in KRAS mutant cancers, like PDAC. Patient sample databases show that an important ER protein, the rhomboid protease RHBDL4, is upregulated in pancreatic cancer. A major role for the active protease RHBDL4 is removing misfolded proteins from the ER and targeting them for degradation by the proteasome, mitigating the toxic stress associated with accumulated misfolded proteins. This stress, when at high levels, can trigger apoptosis (programmed cell death), which cancer cells aim to avoid. In human PDAC cell lines, we made an RHBDL4 knockout (KO) and found that there is

a drastic decrease in cell proliferation and cell migration in the absence of RHBDL4. However, little about what role RHBDL4 plays in PDAC cell health is known. Thus, we aim to identify the role of RHBDL4 PDAC cells and how this pathway drives tumor progression. We hypothesize that PDAC cells upregulate RHBDL4 to compensate for high levels of misfolded proteins, exploiting RHBDL4's quality control function to avoid ER stress driven apoptosis.

P1181/B185

Substrate selection of the yeast signal peptidase complex.

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Secretory proteins are critically dependent on the correct processing of their signal sequence by the signal peptidase complex (SPC). This step, which is essential for the proper folding and localization of proteins in eukaryotic cells, is still not fully understood. In eukaryotes, the SPC comprises four evolutionarily conserved membrane subunits (Spc1-3, Sec11). Here, we investigated the role of Spc2, examining SPC cleavage efficiency on various model and natural signal sequences in yeast cells depleted of or with mutations in Spc2. Our data show that selection of substrates and identification of the cleavage site by SPC is compromised when Spc2 is absent or mutated. Molecular dynamics simulation of the yeast SPC AlphaFold2-Multimer model indicates that membrane thinning at the center of SPC is reduced without Spc2, suggesting a molecular explanation for the altered substrate recognition properties of SPC lacking Spc2. These results provide new insights into the molecular mechanisms by which SPC governs protein biogenesis.

P1182/B186

A β-coronavirus nonstructural protein 6 hijacks translocation machineries into replication centers.

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RNA viruses have developed clever strategies to evade host immune detection and replicate their genomes in relative secrecy. One such strategy involves the biogenesis of replication centers (RCs) on cytoplasmic host organelles, with the endoplasmic reticulum (ER) being a key site. RCs act in this way as membrane "cloaks," allowing viruses to hijack the host's translational machinery. In the case of mouse hepatitis virus (MHV), a model for studying β-coronaviruses, RC formation is driven by the membrane remodeling functions of viral transmembrane non-structural proteins (nsp) 3, 4, and 6. This study focuses on two main objectives: first, to investigate how these nsps rearrange ER membranes to create RCs capable of supporting viral replication, and second, to assess how RC formation impacts resident ER proteins. We show that co-expression of nsp3, nsp4, and nsp6 leads to their co-localization at distinct membrane structures, a process dependent on nsp6. Notably, these RCs generate unique membrane subdomains, while the broader ER network remains largely intact. Interestingly, these subdomains recruit mammalian and multipass translocons—multi-protein complexes responsible for inserting membrane proteins into the ER. Intriguingly, nsp3, 4, and 6 each have multiple transmembrane domains that could be inserted by these translocons. This points to a potential mechanism by which nsp6 organizes the membrane structures formed by nsp3 and nsp4. As expected, these nsp6-induced subdomains do not enrich for translocons that insert tail-anchored proteins, since none of the viral membrane proteins exhibit a tail-anchored topology. In contrast, these subdomains also do not enrich for the canonical machinery that typically delivers substrates to the mammalian translocon. This

observation suggests a different targeting mechanism is at work or that targeting machinery is unnecessary due to the proximity of viral RNA replication and nsp6 subdomains. Our ongoing research aims to explore how the enrichment of translocon machinery contributes to RC formation and function, with the goal of understanding how these membrane microenvironments support viral replication and potentially facilitate viral assembly.

P1183/B187

The Ufmylation System Plays an Important Role in Forward Delivery and Segregation of Nascent Membrane Proteins at the Endoplasmic Reticulum.

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Ufmylation is a ubiquitination-like posttranslational modification in which ubiquitin-fold modifier 1 (UFM1) is conjugated to the targeted proteins through the E1 UFM1 activation enzyme 5 (UBA5), the E2 UFM1-conjugating enzyme 1 (UFC1) and the E3 UFM1-specific ligase 1 (UFL1). UFM1 on the ufmylated proteins can be cleaved by UFM1-specific protease 1 and 2 (UFSP1 and UFSP2). This system is implicated in multiple cellular processes and human diseases, but little is known about its functions and regulation in protein trafficking. Here, we demonstrate that the CRISPR-mediated depletion of each of the core components in the ufmylation cascade, including UFM1, UBA5, UFL1, UFSP2, and UFM1-binding protein 1 (UFBP1) markedly inhibits the transport of a subset of G protein-coupled receptors (GPCRs) from the endoplasmic reticulum (ER) to the Golgi. More interestingly, UFBP1 localizes at specific Sec24-coated COPII vesicles and both UFBP1 and UFL1 are known to form a complex interact with GPCRs they regulate. Furthermore, the UFBP1/UFL1-binding domain identified in the GPCRs is able to effectively convert non-GPCR protein transport into the ufmylation-dependent route. Collectively, these data identify important functions for the ufmylation system in the biosynthetic transport and sorting of membrane proteins, specifically GPCRs, at the ER level.

P1184/B188

Nutrient Deprivation Alters the Rate of COPII Subunit Recruitment at ER Subdomains to Tune Secretory Protein Transport.

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Co-assembly of the multilayered coat protein complex II (COPII) with the Sar1 GTPase at subdomains of the endoplasmic reticulum (ER) enables secretory cargoes to be concentrated efficiently within nascent transport intermediates. Here, we define the spatiotemporal accumulation and mobility of native COPII subunits and secretory cargoes at ER subdomains under differing nutrient availability conditions using a combination of CRISPR/Cas9-mediated genome editing and live cell imaging. Our findings demonstrate that the rate of inner COPII coat molecule recruitment serves as a determinant for the pace of cargo molecule export, irrespective of COPII subunit expression levels. Moreover, increasing inner COPII coat recruitment kinetics is sufficient to rescue cargo trafficking deficits caused by acute nutrient limitation. Our findings are consistent with a model in which the rate of inner COPII coat addition acts as an important control point to regulate cargo export from the ER.

P1185/B189

Negative and positive regulation of SNARE activity in ER-Golgi transport.

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SNARE-mediated fusion is essential for protein transport from the endoplasmic reticulum to the Golgi. Four SNAREs are required for trans-complex formation, producing the energy to initiate fusion. The coatomer protein complex II (COPII) recruits SNAREs into transport intermediates at the transitional ER. Using a chemically defined in vitro system, our goal is to reconstitute the complete set of subreactions from COPII carrier formation to Rab-mediated tethering and fusion at the target membrane. The COPII coat has been reported to both inhibit and stimulate fusion at target membranes. We found that the COPII inner shell complex (Sec23, Sec24, and Sar1:GTP) potently inhibits fusion, while the outer shell (Sec13 and Sec31) is not required for this inhibition. Sec24 mutants that can assemble and bud vesicles, but that cannot bind SNARE proteins, alleviated this inhibition of fusion, even in the presence of Sar1 bound to a non-hydrolyzable GTP analog. A SNARE chaperone essential for fusion, the Sec1/Munc-18 protein Sly1, directly interacts with SNAREs and membranes to promote short-range tethering and fusion (1,2). Our preliminary data support a working model in which Sly1 enforces a requirement for the Qa-SNARE Sed5 and the R-SNARE Sec22 to operate in trans. The relative orientation of the other two SNAREs does not appear to affect fusion activity. Ongoing experiments aim to understand how long range tethers such as Uso1/p115 hand off SNAREs to Sly1 to initiate short range tethering and membrane fusion.

1. Duan M*, Plemel RL*, Takenaka T, Lin A, Delgado BM, Nattermann U, Nickerson DP, Mima J, Miller EA, Merz AJ. 2024. SNARE chaperone Sly1 directly mediates close-range vesicle tethering. Journal of Cell Biology, doi: 10.1083/jcb.202001032. *Equal contribution.2. Duan M, Gao G, Lin A, Mackey EJ, Banfield DK, Merz AJ. 2024. SM protein Sly1 and a SNARE Habc domain promote membrane fusion through multiple mechanisms. Journal of Cell Biology, doi: 10.1083/jcb.202001034.

P1186/B190

Investigating *mia2/mia3*function in zebrafish photoreceptors.

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From a mutational screen in zebrafish, we identified two related genes that are essential for maintaining age-related photoreceptor health, *mia2* and *mia3*. Of significance, mutations in human and dog MIA3 have recently been shown to cause retinopathies. How the splice isoforms of *mia2* (Ctage5 and Tali) and *mia3* (Tango1L and Tango1S) affect photoreceptor health is uncharacterized. Mechanistically, *mia2/mia3* protein isoforms act at ER exit sites to expand the budding COPII vesicle and facilitate large cargo secretion to the Golgi. Photoreceptor health is dependent upon intrinsic and extrinsic large proteins. Disrupted secretion of several of these large proteins has been shown to cause photoreceptor degeneration. Additionally, disruption of unconventional protein secretion, which circumvents the Golgi, can also cause photoreceptor degeneration. Unconventional protein secretion in photoreceptors occurs through poorly defined mechanisms. We hypothesize *mia2/mia3* function within photoreceptors to accommodate secretion of large proteins, but also plays a role in the unconventional secretion pathway. To test this hypothesis, we assessed photoreceptors in *mia2/mia3* mutants created using Crispr Cas9 deletions. Both *mia2* (Ctage5/Tali double knockout) and *mia3* (Tango1S and Tango1S/Tango1L double knockout) mutants resulted in aberrant photoreceptor morphology and degeneration. Interestingly, Tango1L mutants did not result in disruptions to photoreceptors. Our results validated previous research

that shows altered protein secretion in photoreceptors leads to degeneration and underlies the importance of *mia2/mia3* in photoreceptor health. Future experiments will evaluate all isoforms of *mia2/mia3* by immuno-based markers and track fluorescently tagged candidate proteins. In addition, we will apply an in vivo protein proximity labeling assay (TurboID) to identify Mia2/Mia3 interactants within the photoreceptors to identify proteins involved in unconventional or large protein secretion. Finally, planned experiments include conditional gene deletions and expression of cell-type specific transgenic peptide inhibitors of *mia2/mia3* to determine cell autonomous effects.

P1187/B191

Regulation of ER Exit for Ceramide Metabolism and Trafficking.

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Cells establish organelle membrane homeostasis via regulation of lipid metabolism and trafficking. Membrane lipids are comprised of more than ten thousand lipid species, possessing distinctive physical property and biological functions. Among them, complex sphingolipids such as sphingomyelin (SM) and cholesterol are known to concentrate in the plasma membrane (PM), binding each other and contributing to the high rigidity in the PM. Interestingly, loss of cholesterol activates the synthesis of SM, suggesting an undiscovered, intricate metabolic relationship between SM and cholesterol. Two types of sphingolipids are significant in cellular quantity and biological roles; sphingolipids containing 14-to-20 carbon fatty acids called long-chain (LC) sphingolipids and those containing 22-26 carbon fatty acids called very-long-chain (VLC) sphingolipids. The chain-length is biochemically determined by specific ceramide synthases (CerS1 to 6) that produce ceramides, the precursor of SM and other complex sphingolipids. We previously show that cholesterol depletion activates the synthesis of only one lipid type, VLC-SM, and that the synthesis of VLC-SM depends not only on the enzymes CerS and SM synthase (SMS) but also on COPII-dependent ER-to-Golgi secretory pathway. Here we demonstrate that cholesterol depletion likely activates VLC-ceramide trafficking via activating ER exit. We found that, via metabolic flux analysis, the level of substrate, VLC-ceramide, was reduced and the synthesis of the product, VLC-SM, was increased upon cholesterol depletion. Cells lacking SMS1 and 2 showed increased level of VLC-hexosyl-ceramides, another major complex sphingolipid. The data strongly suggest that the substrate ceramide trafficking is activated upon cholesterol depletion. We used in situ Click reaction to visualize clickable sphingolipids provided in cells and observed that the sphingolipids reached to the Golgi apparatus faster when cells were deprived of cholesterol. This difference was diminished when cells lack CerS2, the major enzyme making VLC-ceramide. We hypothesize that ER exit is activated by loss of cholesterol. We observed that, by immunofluorescence microscopy, the size of Sec16A, the platform for the recruitment of ER exit components, were reduced upon cholesterol depletion. Because the bigger size of Sec16A has been associated with reduced ER exit functions, our data suggest that loss of cholesterol initiates cellular signaling that activates Sec16A and ER-exit, and thus increasing VLC-ceramide trafficking and synthesis of downstream VLC-SM. Our data show how cells regulate VLC-ceramide trafficking and downstream metabolism upon changes in cholesterol level.

P1188/B192

The zinc metalloprotease ZMPSTE24 binds a specific topological isoform of the antiviral tail anchor protein IFITM3.

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ZMPSTE24 is a multi-spanning membrane zinc metalloprotease with an HEXXH catalytic motif. ZMPSTE24 localizes to the ER and inner nuclear membranes and has an established role in the proteolytic processing of prelamin A, precursor of the nuclear scaffold protein lamin A. Although prelamin A is ZMPSTE24's only well-defined substrate, both ZMPSTE24 and its yeast homolog Ste24 have also been shown to play an ill-defined role in the ER quality control of prematurely folded secretory proteins which clog the Sec61 translocon, disrupting translocation into the ER lumen. The interferon-induced transmembrane proteins (IFITM1, 2, and 3) are antiviral restriction factors that block infection of enveloped viruses by inhibiting the fusion of virus and host cell membranes, likely by rigidifying cellular membranes. The IFITMs have a C-terminal transmembrane span (tail-anchor) and three palmitoylation sites. Recently, ZMPSTE24, like the IFITMs, was shown to reduce infection of enveloped viruses and to interact with the IFITM proteins, although the functional significance of this interaction is not well-understood. Here, we show that a catalytic-dead version of ZMPSTE24, ZMPSTE24^{E336A}, enhances IFITM3 binding, potentially trapping either a normally transient biogenesis intermediate or an incorrectly inserted isoform of IFITM3. ZMPSTE24^{E336A} binds hypo-palmitoylated IFITM3 and requires specific cysteines for these interactions. Using a split fluorescence protein reporter, we show that ZMPSTE24^{E336A} expression stabilizes a membrane topology of IFITM3 not observed in control cells. We hypothesize that ZMPSTE24 plays a role in protein quality control by the removal of transmembrane proteins that do not attain their correct membrane topology.

P1189/B193

Determination of Subcellular Localization and Membrane Topology of Phospholipase ABHD3.

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Alpha/beta-hydrolase domain containing 3 (ABHD3) is a phospholipase that releases free fatty acids from phospholipids, crucial components of biological membranes. ABHD3 shows strong affinity/activity for myristoyl (14:0) phosphatidylcholine and phospholipids with oxidatively truncated acyl chain resulting from oxidative stress. Also, ABHD3 is highly expressed in breast cancer. Despite its unique properties, the physiological role of ABHD3 in the cells remains unclear. Furthermore, the subcellular localization of ABHD3 has yet to be fully characterized through experimental studies although the localization critically contributes to the function. In this study, we aimed to clarify the function of ABHD3 by detailing its subcellular localization. Based on its amino acid sequence, ABHD3 is suggested to be a single-pass membrane protein. Immunofluorescent staining using the cells overexpressing C terminal FLAG-tagged ABHD3 (ABHD3-FLAG) revealed a reticular fluorescence pattern in the cytoplasm, perfectly overlapped with the fluorescent pattern of stained calnexin, an endoplasmic reticulum (ER) protein. Of special note is that the fluorescent pattern of ABHD3-FLAG did not overlap with the maker for plasma membrane and mitochondria. These results revealed that ABHD3 specifically localizes to the ER. To confirm the transmembrane (TM) region of ABHD3, the cell overexpressing ABHD3-FLAG lacking the predicted TM region (amino acid residue 27-54) was checked. As we expected, ABHD3 lacking TM region showed no specific localization and dispersed in the cytosol. These results unveiled that ABHD3

possesses its catalytic domain at the C terminal of the TM region. Thus, we sought to determine the catalytic domain of ABHD3 faces to either the outer or inner layer of the ER membrane using several online membrane topology prediction tools. However, definitive results were not obtained. Therefore, we employed the fluorescent protein proteinase protection (FPP) assay (Lorenz, H. *et al. Nat. Methods* **3** 205-210, 2006) using C terminal GFP-tagged ABHD3 to determine the membrane topology. The fluorescence from ABHD3-GFP at ER was diminished by proteinase treatment following plasma membrane permeabilization. Indicating that ABHD3 is a type 1 transmembrane protein with its catalytic domain facing the outer layer of the ER membrane. This study demonstrated that ABHD3 may contribute to the ER membrane phospholipids' metabolism at the outer layer and provides a key feature for elucidating the physiological functions of ABHD3 in the cells. We are trying to elucidate the function of ABHD3, especially focusing on the ER-related cellular responses.

P1190/B194

Investigating the role of SPTLC1 variant C133W in ERAD regulation.

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Cells face a myriad of stressors that can trigger the aggregation of misfolded proteins, known pathological hallmarks for neurodegenerative diseases, aging, and cancer. However, there are quality control mechanisms to protect from the dangers of misfolded proteins, such as Endoplasmic Reticulum-Associated Degradation (ERAD). This pathway consists of identification and ubiquitination of misfolded endoplasmic reticulum (ER) proteins, and extraction (or retrotranslocation) of these proteins from the ER to the cytosol for degradation by the cytosolic proteasome. Retrotranslocation is mediated by transmembrane rhomboid pseudoproteases Derlins in mammals, and Dfm1 in yeast. Our lab has shown that Dfm1 thins the ER lipid membrane to facilitate retrotranslocation of membrane substrates. In addition to playing a key role in ERAD retrotranslocation, we also show that Dfm1 contains an ERAD-independent role of mediating sphingolipid homeostasis by associating with serine palmitoyltransferase (SPT) enzymes, which play a key role in the rate limiting step of sphingolipid biosynthesis and regulation. Sphingolipids are key structural components of membranes and are important regulators for signaling and stress responses. When one of the SPT subunits SPTLC1 contains a disease-associated mutation C133W, the SPT complex metabolizes alanine rather than serine, which produces deoxysphingolipids. Our collaborators have shown that, when overexpressing SPTLC1 C133W in human retinal pigment epithelial cells, 1) deoxysphingolipids accumulate at the ER and 2) ER lipid membrane is more rigid compared to that of wildtype. Given our and others findings that lipid composition affect ERAD retrotranslocation, we hypothesize that SPTLC1 C133W dysregulates ERAD compared to wildtype SPTLC1. Currently, we show that SPTLC1 C133W 1) increases protein expression of human Derlin-2 rhomboid pseudoprotease and 2) stabilizes the degradation of an ERAD substrate ORMDL3, implicating that accumulation of deoxysphingolipids within the ER negatively impacts ERAD. Additional biochemical approaches and microscopy will aid in determining the connection between deoxysphingolipid production and ERAD regulation. SPTLC1 C133W is a well-documented variant found in patients with hereditary sensory and autonomic neuropathy (HSAN1), and considering that accumulation of misfolded proteins are hallmarks for neurodegenerative diseases, deciphering the relationship between SPTLC1 C133W and ERAD will provide invaluable insight and foundation for therapeutic design.

P1191/B195

Direct visualization of retrotranslocation by the Hrd1 complex by high speed single molecule tracking in living cells.

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Terminally misfolded or otherwise damaged proteins of the Endoplasmic Reticulum (ER) are degraded by the proteasome in a process termed Endoplasmic Reticulum Associated Degradation (ERAD). The Hrd1 complex is a multicomponent transmembrane protein complex that mediates ubiquitination and export of proteins from the ER to be degraded in the cytosol. Despite substantial effort in the past two decades, the biochemical characterization of its architecture and mechanism produced inconsistent and even contradictory results, yielding no consensus on how it mediates protein transport. Its elusive nature is representative of the limitations of classical biochemical approaches, whose often harsh experimental conditions directly interfere with the objects they study. In this project we used fluorescence multi-color single molecule microscopy to offer a new perspective on the architecture, formation and dynamics of the Hrd1 complex. In this process, we developed cell biological, experimental and analytical tools to robustly quantify and characterize Hrd1 oligomerization *in vivo*. Combining live-cell dual-color single-particle tracking with chemical inhibition, downregulation of complex components and a novel, binding-competition based tracking assay, we demonstrated that Hrd1 forms a stable homo-tetramer via its cytosolic domain $Hrd1_{480\text{-}529}$. By structural modeling via AlphaFold, results of which were validated with both single-particle tracking and recombinant protein expression, we showed that this domain assembles into a canonical coiled-coil domain independently of other complex components or Hrd1's activity. While yielding specific novel biological insight into Hrd1 complex formation, it also serves as a general blueprint on how dual-color single particle tracking can be used to address questions that bring classical biochemistry to its limits.

P1192/B196

Deciphering the molecular mechanism and downstream effects of the endoplasmic reticulum stress sensor IRE1.

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The endoplasmic reticulum (ER) is a large membrane-bound organelle that serves critical functions in eukaryotic protein quality control and protein synthesis. An intricate intracellular signaling network termed the Unfolded Protein Response (UPR) performs constant feedback on the ER to maintain it in a healthy, balanced state. The UPR comprises three upstream transmembrane sensors that exhibit distinct spatiotemporal regulation and work together to execute complex transcriptional and translational programs. Such intertwined signaling makes it difficult to resolve the roles and signaling mechanisms of each individual UPR sensor. We tackled two major outstanding questions: 1) How is the human ER stress sensor Inositol-Requiring Enzyme 1 alpha (IRE1) activated at a molecular level? and 2) What is the cellular response to “pure” IRE1 activation in the absence of all other stress signaling? We addressed these questions with a multi-pronged approach combining live-cell and *in vitro* optogenetics, single-molecule live-cell imaging, long-read sequencing, and precision enzymology. Our imaging results suggest that IRE1’s primary regulatory transition is that between inactive dephosphorylated dimers and active phosphorylated dimers, while *in vitro* enzymology with precise control over IRE1’s oligomeric state

offers a mechanistic insight into the activation of IRE1's RNase domain in response to both dimerization and phosphorylation. Long-read transcriptomic analysis of optogenetically controlled IRE1 in human cells reveals a more subtle and targeted response than the previously characterized "acute" UPR that is elicited by potent chemical perturbations of the ER lumen. This targeted response may help explain IRE1's specific role in continuously controlling the size and composition of the ER in healthy cells, in the absence of severe proteostatic challenges. Since IRE1 and other UPR sensors are differentially expressed and regulated both during development and in the adult organism, understanding how IRE1 functions without interference from other branches of the UPR is necessary for delineating its broader role in cellular homeostasis.

P1193/B197

Endoplasmic Reticulum Homeostasis Maintenance by ERK5 is Crucial for Early Porcine Embryonic Development.

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Extracellular signal-regulated kinase 5 (ERK5), a mitogen-activated protein kinase (MAPK) family member, plays an important role in various biological processes, such as proliferation, apoptosis, differentiation, survival, and cell regulation. However, studies on the effects of ERK5 on porcine preimplantation embryos are limited. In this study, to determine the function of ERK5 during porcine embryo development, ERK5 function was inhibited by adding the ERK5 inhibitor JWG-071. The ERK5 mRNA and protein expression levels tended to decrease from the 4-cell stage compared to the 1-cell and 2-cell stages, suggesting that ERK5 is the maternal gene. During embryonic development in pigs, adding 5 μM of JWG-071 significantly reduced the phosphorylation of ERK5 and the blastocyst development rate (control: 53.44 ± 8.38%; treatment: 26.65 ± 3.40%). Additionally, ERK5 inhibition increased the expression of UPR-related proteins, glucose-regulated protein (GRP78), and C/EBP homologous protein (CHOP) by inducing ER stress. Compared to the control group, the expression of the autophagy-related proteins LC3 and *ATG7* was significantly increased in the ERK5 inhibition group, indicating that the inhibition of ERK5 induced autophagy. In addition, ERK5 inhibition increased the expression of *BAX*, a pro-apoptotic gene, resulting in apoptosis. In conclusion, the results show that ERK5 inhibition during porcine embryonic development induces autophagy and apoptosis by increasing ER stress, resulting in a negative effect on embryonic development in pigs.Keywords: MAPK, Autophagy, Apoptosis, ER stress, UPR

P1194/B198

A Novel ERAD-to-mTORC1 Signaling Axis Constitutes a Vulnerability of Multiple Myeloma Cells to Targeting Secretory Proteostasis.

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Multiple myeloma (MM), the second most common hematological malignancy, results from the clonal proliferation of malignant plasma cells in the bone marrow. MM cells produce and secrete vast amounts of immunoglobulins, requiring major adaptive changes of the endoplasmic reticulum (ER) proteostasis machinery. Our study aims to identify vulnerabilities within the ER-export machinery as therapeutic targets for MM. Secretory proteins leave the ER in a COPII-dependent manner at ER exit sites (ERES).

The four SEC24 paralogs (A-D) are the part of the COPII coat responsible for capturing secretory cargo. Notably, the machinery controlling immunoglobulin trafficking from the ER are poorly investigated, and the impact of disrupting this process on myeloma cell survival remains unclear. We performed single and combined siRNA knockdowns of SEC24 paralogs in non-secretory MM cells (KMS-12PE) and secretory MM cells (AMO-1, and NCI-H929). While the non-secretory KMS-12PE cells were insensitive, both secretory cells lines were highly sensitive to silencing SEC24 paralogs, particularly SEC24A&B. Accordingly, immunoglobulins exhibited stronger colocalization with SEC24A&B-positive ERES. Interestingly, although knockdown of SEC24A&B increased misfolded protein levels, we did not observe a major increase of the UPR, indicating that ER stress is not responsible for sensitizing secretory MM cells to death. Instead, SEC24A&B silenced cells exhibited higher mTORC1 activity, and inhibition of mTORC1 rescued these cells from death. This indicates a link between ER-export disruption and mTORC1 signaling. Silencing SEC24A&B increased the level of misfolded proteins in the ER, inducing ERAD-mediated proteasomal protein degradation, thereby increasing cytosolic amino acid levels. Inhibiting ERAD rescued SEC24A&B knockdown MM cells from death. Notably, despite higher mTORC1 activity, the translation rate was significantly lower in SEC24A&B depleted cells, indicative of metabolic imbalance, where a higher energetic demand cannot be met. Altering ER-export resulted in swelling of mitochondria, reduced the spare respiratory capacity and a reduced ability to produce ATP, thus explaining why the higher energetic demand cannot be met. Altogether, our work reveals an unprecedented link between ERAD and mTORC1 signaling that can be exploited a potential therapeutic strategy in multiple myeloma.

Connecting Organelles: Contact and Communication

P1195/B199

BLTP2/KIAA0100, a Bridge-like Lipid Transfer Protein, is enriched at contacts between the endoplasmic reticulum and recycling membranes upon their fusion with the plasma membrane.

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Bridge-Like Lipid Transfer Proteins (BLTPs) are a newly identified family of rod-shaped proteins, comprising repeating β-groove (RBG) motifs and thought to mediate unidirectional bulk transport of membrane lipids between two closely apposed membranous organelles. They comprise VPS13, the founding member of the family, ATG2 and three other less characterized proteins: BLTP1, BLTP2and BLTP3. Here, we have explored the cellular function of human BLTP2/KIAA0100, a large protein anchored in the endoplasmic reticulum (ER) via an N-terminal transmembrane helix. While some previous studies of BLTP2 had reported its localization at ER-plasma membrane (PM) contacts (Neuman et al., PMID:34415038, Banerjee et al., PMID: 38370643), another study found BLTP2 at the tips of tubular recycling endosomes, but not at ER-PM contacts (Parolek and Burd, PMID: 38535441). Here we reconcile the two sets of findings by showing that the tips of Rab10 positive tubular endosomes which are positive for BLTP2 are continuous with the PM. Formation of these contacts is regulated by phosphoinositide PI4P and by BLTP2-binding proteins including N-BAR protein BIN1 and FAM102A/FAM102B. Moreover, we found that BLTP2 is also recruited to contacts of the ER with recycling macropinosomes undergoing fusion with the PM. The knock-out of BLTP2 results in a perturbation of the dynamics of these PM-connected structures. In view of a recent study suggesting

that BLTP2 may help deliver lipids that increase fluidity of the PM (Banerjee et al., PMID: 38370643), we hypothesize that this protein may play a role in the control of the lipid composition of recycling membranes as they fuse with the plasma membrane.

P1196/B200

Genetic analysis of Endoplasmic reticulum (ER) - Plasma membrane (PM) junction in neurons.

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Endoplasmic reticulum (ER) - Plasma membrane (PM) junctions are organelle-organelle junctions between the most abundant endomembrane system (ER) and the PM. At the junctions, the two membranes are in close association, with an interspace ranging from 7 to 30 nm where a set of ER transmembrane and cytosolic proteins with phospholipid binding properties reside. The ER-PM junctions are present broadly in eukaryotes. It plays important functions in Ca2+ regulation, signaling and lipid exchange. Their abundance and morphology greatly vary from cell to cell and can be modulated by the functional state. Many important questions in the structure and function of ER-PM junctions remain incompletely understood. The inventory of proteins mediating ER-PM junction remains far from complete. Little is known about the mechanisms that regulate the shape, size and abundance of ER-PM junctions. Furthermore, excitable cells like neurons contain abundant, specialized ER-PM junction compared with non-excitable cells. Development and regulation of neuronal ER-PM junctions is poorly understood. Lastly, abnormal ER-PM crosstalk has been implicated in neurological disorders including Amyotrophic Lateral Sclerosis, Parkinson's disease, and Huntington disease. To understand cellular mechanisms that regulate the size, shape and abundance of ER-PM junctions in neurons, I visualized ER-PM junctions in the C. elegans PVD neuron with a GFP::MAPPER marker. Confirmed by electron microscopy (EM) reconstruction, the ER-PM junctions are patch-like intermembrane structures evenly distributed on the PM in a “soccer” pattern. I performed both forward genetic and reverse genetic approach to identify mutants with abnormal ER-PM junctions. From these mutants, I discovered that the size and shape ER-PM junctions requires a number of genes including microtubules (MTs), a cortical MTs anchoring complex, Kinesin 1/UNC-116. Furthermore, phosphatidylinositol 4-kinase alpha (PI4KA) regulates ER-PM junction abundance, consistent with the presence of numerous phospholipid binding proteins at the junction. The goal of the studies is to provide conceptual understanding of ER-PM junction morphogenesis in neuron and to test whether these mechanisms are conserved in vertebrate system by studying their vertebrate homologs.

P1197/B201

Multiple cAMP/PKA Complexes at STIM1 ER/PM Junctions, Specified by E-Syt1 and E-Syt2, Reciprocally Gate ANO1 (TMEM16A) via Ca2+.

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IRBIT (Inositol 1,4,5-Trisphosphate (IP_3) Receptor Released with IP_3), an inhibitor of the IP_3 receptors, also regulates several transporters, to coordinate neuronal function and secretory glands fluid secretion. Our present work focus on whether and how IRBIT affects channels function *in vivo* and *in vitro* to determine the physiological fluid secretion. Our findings indicated that IRBIT mediates epithelial

transport in vivo via expanding signaling repertoire by targeting the transporters to different adenylyl cyclases (ACs) at selective ER/PM junctional domain with the aid of the extended synaptotagmins (E-Syts) E-Syt1 and E-Syt2. This results in a synergy between cAMP and Ca^{2+} signaling to widen and buffer the regulation of the Ca^{2+}-activated Cl^- channel by ANO1(Anoctamin 1, TMEM16A), which has been identified as a decision-making channel to control epithelial fluid and electrolyte secretion. We reported that knockout of IRBIT enhances the receptor-stimulated-Ca^{2+} signaling, yet receptor-stimulated ANO1 activity is reduced in IRBIT$^{-/-}$ mice. The paradoxical finding *in vivo* was traced to IRBIT regulation of ANO1 through two independent mechanisms. First, IRBIT increased the surface expression of ANO1 and thus raised its current density. Second, IRBIT recruits ANO1 to the E-Syt1 assembled ER/PM junctions containing AC8/AKAP79/PKA to sensitize activation of ANO1 by Ca^{2+} and to the E-Syt2 assembled containing AC6/AKAP11/PKA to desensitize activation of ANO1 by Ca^{2+}. The sensitization/desensitization effects involves phosphorylation of selective serine residues. Disruption of the ER/PM junctions by deletion of the E-Syts or STIM1 eliminated PKA-dependent IRBIT-dependent ANO1 regulating, which the opposite observe upon increasing the junction numbers. These findings indicated that ER/PM junctions were essential for synergy in signaling and IRBIT uses the cAMP/PKA system to widely expand and buffer activation of ANO1 by Ca^{2+} and thus critically control the physiological response.

P1198/B202

ER-Plasma Membrane Contact Sites Deliver ER Lipids and Proteins for Rapid Cell Surface Expansion.

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15.00 Normal 0 false false false EN-US X-NONE X-NONE /* Style Definitions */
table.MsoNormalTable{mso-style-name:"Table Normal";mso-tstyle-rowband-size:0;mso-tstyle-colband-size:0;mso-style-noshow:yes;mso-style-priority:99;mso-style-parent:"";mso-padding-alt:0in 5.4pt 0in 5.4pt;mso-para-margin:0in;mso-para-margin-bottom:.0001pt;mso-pagination:widow-orphan;font-size:12.0pt;font-family:"Calibri",sans-serif;mso-ascii-font-family:Calibri;mso-ascii-theme-font:minor-latin;mso-hansi-font-family:Calibri;mso-hansi-theme-font:minor-latin;mso-bidi-font-family:"Times New Roman";mso-bidi-theme-font:minor-bidi;mso-font-kerning:1.0pt;mso-ligatures:standardcontextual;}Rapidincreases in cell surface area occur often, which can be the consequence ofchanging cell shape (e.g., fast migrating cells) or hypoosmotic shock thatcauses swelling of the cell. How cells maintain plasma membrane (PM) integrityduring these rapid surface expansions is not known. To get a betterunderstanding of this phenomenon, we used yeast *Saccharomyces cerevisiae*to identify proteins required to maintain cell integrity during hypoosmoticswelling of the cell. A candidate approach using a cytoplasmic pH indicator asa readout for cell integrity identified the tricalbins Tcb1, Tcb2 and Tcb3 ascritical factors in maintaining PM barrier function during rapid cellexpansion. The tricalbins are calcium-binding proteins of the ER-PM contactsites that have been implicated in lipid transport between the two membranes.We found that these proteins allow for 1% cell surface increase per second,which corresponds to ~4,000,000 lipids transported from the cortical ER to thePM every second. Most interestingly, we also identified a GPI-anchored proteinthat during cell expansion was rapidly delivered from the ER to the cellsurface. These observations suggested that hypoosmotic shock caused a directfusion of the ER with the PM at contact sites, allowing for both lipids andproteins to be delivered to the expanding PM. The tricalbins are calciumbinding proteins and we found that the stretch-activated calcium channel Cch1and external calcium was necessary for the proposed ER-PM fusion. Together, ourdata suggest that high tension of the PM triggers the opening of Cch1, allowingfor the influx of extracellular calcium. Calcium binds to the tricalbins, whichbased on

previous studies pull ER and PM membrane at contact sites closertogether. As a consequence, the membranes fuse, delivering lipids and proteinsto the cell surface, thereby preventing the rupture of the rapidly expandingPM.

P1199/B203

The Novel Role of Endoplasmic Reticulum-Plasma Membrane Contact Sites in Expression of High-Mannose Integrins in Advanced Prostate Cancer.

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Integrins are crucial in cancer progression and metastasis but targeting them is challenging due to the complexity of integrin subtypes and N-glycan modifications. We have demonstrated that Golgi fragmentation, ER stress signaling, and autophagy collectively increase pro-metastatic Integrin α_v expression on the plasma membrane (PM) of advanced prostate cancer (PCa) cells. Specifically, we reported that Integrin and Galectin-3 (Gal-3) are elevated on the cell surface in high-grade PCa tissues: Golgi fragmentation leads to abnormal N-acetylglucosaminyltransferase-V (MGAT5)-mediated glycosylation of integrins, promoting Gal-3-mediated clustering and retention of integrins on the PM, a key event in prostate tumor metastasis. However, data from our lab and others suggest that underglycosylated, high-mannose (high-Man) integrins are also linked to more severe PCa, though the underlying mechanism and implications are unclear. We studied the role of ER-PM junctions and Golgi morphology in such glycosylation of α_v-integrins, using siRNA-mediated knockdown (KD) of ER-resident proteins, Stromal Interaction molecule 1 (STIM1) and oxysterol binding protein like 5 (ORP5), individually and together. High-resolution and electron microscopies revealed that these KDs reduced the overall amount of Integrin α_v on the cell surface. Furthermore, co-KD of STIM1 and ORP5 significantly diminishes Golgi disorganization, decreasing high-Man integrins on the PM. Also, co-KD reduced Gal-3 on the PM while increasing its colocalization with Integrin on the PM. A similar response was found in cells lacking critical ER stress mediator ATF6. We hypothesize that high-Man integrins may utilize an alternative trafficking pathway as a protective mechanism to reduce MGAT5-mediated pro-metastatic glycosylation. Notably, cells lacking both STIM1 and ORP5 showed increased fibronectin adhesion despite reduced PM-localized Integrin, suggesting enhanced adhesion of PCa cells to the extracellular matrix, a crucial step in cancer cell spreading. Notably, the expression of high-Man integrins was significantly reduced in metastatic tumor samples, supporting our concept. This study is the first to provide evidence that high-Man glycan expression in cancer cells may be triggered by ER stress and Golgi disorganization and driven by ER-PM junctions, representing a potential anti-tumor mechanism.

P1200/B204

ATF4 Regulation of MERCs and Cristae Morphology in Skeletal Muscle.

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Skeletal muscle (SkM) is vital for maintaining physical and metabolic health. A loss of SkM mass, or atrophy, can have debilitating repercussions, significantly decreasing both the quality and quantity of life. Although several biochemical pathways have been associated with SkM atrophy, it is becoming more apparent that these pathways are only a small part of a massive, complex signaling network that remains largely unexplored. Recently, a stress-inducible transcription factor, activating transcription factor 4 (ATF4), has been shown to contribute to SkM atrophy. Under stressed conditions, ATF4

facilitates cellular adaptation, enabling cells to survive under the new conditions. However, prolonged ATF4 activation is maladaptive, promoting muscle atrophy and transcriptional repression of genes involved in mitochondrial function, metabolic processes, and protein biosynthesis. Mitochondria and endoplasmic reticulum (ER) contact sites (MERCs) mediate vital organelle-organelle communication processes, including lipid and calcium (Ca^{2+}) exchange. We recently showed that increased MERC tethering is ATF4-dependent in the absence of OPA1. However, the mechanisms driving these remain unknown which we sought to investigate with this study. We hypothesized that noncanonical ATF4 signaling results in increased MERC tethering, subsequently altering the morphology and function of mitochondria juxtaposed with the ER. To test our hypothesis, we utilized focused ion beam-scanning electron microscopy (FIB-SEM) and 3D reconstruction software to manually segment, analyze and quantify structural changes across different ATF4 expression levels and supplemented this data with standard molecular and biochemical analyses. Here, we show that changes in ATF4 expression alters both mitochondrial and MERC morphologies, proteins involved in MERC Ca^{2+} exchange, and the metabolome in *Drosophila* flight muscles. Additionally, we show that these changes are also prevalent in primary mouse myotubes with altered ATF4 expression levels. Together, these data suggest that altered ATF4 signaling may play an integral role in the altered organelle-organelle communication and mitochondrial ultrastructure observed with SkM atrophy.

P1201/B205

Analysis of a role for mitochondria-associated ER membranes (MAMs) in a specialized cell elimination program.

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Programmed cell death is critical for normal development and homeostasis. Specialized cells are characterized by elaborate processes spanning differing microenvironments, such as axons and dendrites in neurons. While these specialized cells are very common, their programmed elimination is poorly understood, as is their elimination under pathological conditions or following injury. We discovered a 'tripartite' killing program that eliminates the specialized tail-spike cell (TSC) and the sex-specific CEM neurons of the nematode C. elegans during its embryonic development. We named this program Compartmentalized Cell Elimination (CCE), and it is characterized by three cell regions dying in three disparate ways. Particularly, the single process/dendrite of these cells displays two very different elimination morphologies in its two segments, strikingly reminiscent of different types of developmental pruning. The proximal segment fragments in a manner similar to injury-induced Wallerian degeneration of axons, while the distal segment retracts into itself. We report in previous studies that retrograde transport of mitochondria and their subsequent sequestration in the soma are a pre-requisite for CCE. We also report genes promoting endoplasmic reticulum (ER) network stability promote process dismantling during CCE. Mitochondria-Associated ER Membranes (MAMs) are the region of the ER tethered to mitochondria that mediate communication between the two organelles. MAMs are known to be involved in calcium homeostasis, lipid synthesis and cell death signaling. Recent studies also show the connection of MAMs to neurodegenerative diseases. We sought to look at the roles of MAMs in CCE, and in neurite pruning overall. Preliminary results show exaggerated mitochondria when ER network stability is compromised. Using complementary approaches of genetics and advanced microscopy, we plan to further determine the nature and functional output of these exaggerated mitochondria.

P1202/B206

Stearoyl-CoA Desaturase 1 (SCD1) is Required for Endoplasmic Reticulum-Mitochondria Membrane Contact Site (ERMCS) Formation and Dynamics in Cancer.

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In a nutrient scarce microenvironment, cancer cells must alter their normal metabolic program to meet the energetic requirements for cancer progression. Cellular metabolism is organized spatially with dedicated organelles. At the nexus of cellular metabolism sits the mitochondrion. Mitochondria integrate multiple upstream nutrient inputs and transduce downstream metabolic signaling to support cancer cell growth. Additionally, the mitochondrial network must be dynamically regulated in cancer to provide metabolic plasticity necessary for adaptation to energetically unfavorable environments. Mitochondria can achieve this through physical contacts with the ER (ERMCS) mediated by tethering proteins to improve mitochondrial function through exchange of metabolites such as phospholipids. However, we lack mechanistic understanding of how ERMCS are regulated and integrated into cellular metabolism and tumor physiology. To investigate this, we employ a Split GFP based Contact Site sensor (SPLICS) to monitor ERMCS. Using high-throughput drug screens and targeted assays we identified that hypoxia through inhibition of an oxygen-dependent enzyme, Stearoyl-CoA Desaturase 1 (SCD1) is sufficient to decrease ERMCS formation basally and in the presence of ERMCS inducers. SCD1 is an endoplasmic reticulum (ER) resident enzyme and the main enzyme responsible for mono-unsaturated fatty acids (MUFAs) biosynthesis. MUFAs are a specific metabolic requirement of tumor cells to support cell growth. To confirm the role of SCD1 in ERMCS, shRNAs were used to genetically knock-down SCD1, which resulted in a significant inhibition of ERMCS formation. Lipidomic data demonstrate that ERMCS are required to mediate lipid homeostasis. Together, these data suggest a bidirectional regulation of ERMCS and lipids to maintain cellular homeostasis and cancer growth. To further investigate this relationship, synthetic, temporally controlled and chemically induced ERMCS tethers were generated to test the effects of chronic ERMCS formation on cell physiology. Our chemically induced proximity ERMCS systems were sufficient to alter mitochondrial morphology in vitro. Future research directions include assessing whole-cell and organelle-specific metabolic changes upon chronic ERMCS induction, rescuing SCD1 activity with MUFAs, and exploring alternative roles of SCD1 in ERMCS.

P1203/B207

ATUM-SEM and correlative super resolution microscopy reveal complex organelle structure maintained by distinctly localized nonmuscle myosin II within the murine kidney thick ascending limb.

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Our previous work established a crucial role for conventional nonmuscle myosin II paralogs NM2A (Myh9) and NM2B (Myh10) within the mouse kidney thick ascending limb (TAL) epithelium, specifically in transport of cargoes uromodulin (UMOD) and ion cotransporter NKCC2 (Otterpohl et al., 2020). While ER-Golgi trafficking pathways have been traditionally studied using cell culture based methods, we were motivated to explore the relationship between NM2 proteins, cargo transport, and organelle architecture *in situ* in intact mouse kidneys. We utilized the ATUM-SEM method (Kasthuri et al., 2015) followed by manual 3D segmentation to reveal a complex organelle architecture and organization within TAL epithelium. In kidney TAL epithelia, the ER consists of an expansive network of tubules that are

regularly present in the narrow space between mitochondria and the adjacent infoldings of the basolateral plasma membrane. Organelles (ER-mitochondria-PM) and widely distributed vesicles are in close contact with increased density of vesicles observed near the apical membrane. In *Myh9&10* conditional knockout (cKO) kidneys, spherical vesicular bodies are seen in close contact with morphologically abnormal ER with decreased volume. Furthermore, while the plasma membrane (PM) and tight junctions appear intact in *Myh9&10* cKO TAL epithelia, the organization of mitochondria within basolateral membrane infoldings is perturbed and ER-mitochondria-PM contact appears reduced. To correlate organelle structure with protein expression and localization, we established immunostaining methods for resin embedded ultrathin (0.16µm) and thick vibratome (30µm) kidney sections combined with super resolution microscopy. TAL cargoes UMOD and NKCC2 and several resident ER proteins have been visualized with distinct organelle associations. We show basolateral infoldings to be actin-rich structures in which NM2A (MYH9) localizes as distinct puncta in close proximity to outer mitochondrial membrane marked by TOM20 and ER marked by calreticulin. We also observe distinct localization patterns for a number of tight junction proteins, including occludin, ZO-1, and TAL-specific claudins on the actin-rich PM infoldings. Overall, our findings indicate a novel and complex *in situ* ER-mitochondria-PM structural architecture within the TAL epithelia that requires the molecular motor NM2 for its organization and function. Our work substantiates the need for organelle biology within intact organs to reimagine the physiologically relevant functional role(s) played by seemingly well characterized proteins like NM2.

P1205/B209

***In vivo* analysis of the Bridge-like lipid transfer protein VPS-13 in *C. elegans*.**

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Lipid transfer across intracellular membranes is essential for cellular function. VPS13 family proteins belong to a newly identified class of lipid transfer proteins known as Bridge-Like Lipid Transfer proteins (BLTPs). BLTPs are proposed to tether two membranes and to mediate bulk lipid transfer between them through their rod-like core. Loss-of-function mutations of VPS13 proteins in humans cause neurodegenerative or neurodevelopmental diseases. *VPS13A* mutations cause chorea-acanthocytosis and *VPS13C* mutations cause early onset Parkinson's disease. However, the function, mechanism of action and regulation of VPS13A and VPS13C are not fully understood, especially at the organismal level. In mammalian cultured cells, VPS13A is localized at contacts of the ER with either mitochondria or with the plasma membrane, where it binds the lipid scramblase XK. VPS13C is primarily localized at the contacts between the ER and endo/lysosomes. VPS13A and VPS13C have a single ortholog in *C. elegans*, VPS-13. In this study, we have characterized the subcellular localization and function of this protein in living worms. Our results indicate that endogenous VPS-13 predominately localizes at the ER-plasma membrane contacts and plays a role in the development of *C. elegans*' germline. Our findings may provide new insights into the function of VPS13A and into mechanisms of chorea-acanthocytosis.

P1206/B210

Nvj3 regulates phosphatidic acid and diacylglycerol homeostasis at ER-endolysosomal junctions.

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Inter-organelle lipid transport is essential for maintaining proper metabolism, organelle integrity, and overall cell homeostasis. The **SNX-RGS protein family** are inter-organelle tethers associated with lipid metabolism and the neurological disorder, SCAR20. Although there have been many studies implicating these proteins in the maintenance of lipid homeostasis, the mechanism of this action remains unclear. Thus, the objective of this study is to understand the mechanical role of SNX-RGS proteins in maintaining lipid homeostasis. Here, we present evidence that SNX-RGS proteins encode a novel lipid binding domain, and influence phosphatidic acid (PA) and diacylglycerol (DAG) metabolism at ER-endolysosomal contact sites. Using purified **Nvj3**, a "minimal SNX-RGS protein" which encodes only the lipid binding module of the protein family, we find that Nvj3 binds phospholipids, including PA, *in vitro*. Genetic and cell-based assays confirm *NVJ3* exhibits genetic interactions with ***PAH1***, the major resident-ER PA phosphatase, and with predicted lipid transporters, such as ***HOB2***. Using thin layer chromatography of lipids and functional growth assays, I find that the loss of *PAH1*, which typically results in slow growth and the pathological accumulation of phospholipids, can be rescued by the deletion of *NVJ3*. Using a fluorescent DAG biosensor, I find that loss of *NVJ3* leads to accumulation of DAG on the vacuole, which is dependent on the PA phosphatase, ***DPP1*** which is localized to the vacuole surface. Collectively, my data supports the **model** where *Nvj3 transports PA from the vacuole to the ER, such that its deletion leads to the accumulation of PA at the vacuole which can be turned over to DAG by Dpp1, independently of Pah1*. Critically, we also find genetic crosstalk between mammalian *NVJ3* orthologs SNX13/SNX14 and *HOB2* ortholog BLTP2, as well as similar changes in PA/DAG metabolism upon protein knockdown. Together, my data supports a model where orthologs of Nvj3, and perhaps Hob2, maintain cellular lipid homeostasis by facilitating the transport of PA and/or DAG to the ER. Ultimately, this work will contribute to our fundamental understanding of lipid storage and how lipid homeostasis is directed by lipid transport at different and distal inter-organelle contact sites.

P1207/B211

Role of posttranslational modifications on the specificity of VAPB tethering behavior.

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Biochemical processes in spatially distinct organelles must be coordinated to facilitate cellular function. This is largely modulated at sites of direct contact between organelle membranes, which are mediated by a highly conserved set of molecular tethering machinery. Recent work has demonstrated that these tethers can respond and adjust tethering capacity very rapidly to cellular cues through a yet unknown mechanism. Here, we systematically examine the evolutionary conservation of posttranslational modifications (PTMs) in the canonical ER tethering proteins VAPA and VAPB. We couple this to direct observation of the specificity and target of tethering using our recently developed high-speed single molecule tracking pipeline. We examine the capacity of these ER-resident proteins to interact with mitochondria, endosomes, lysosomes, peroxisomes, and the plasma membrane. These results demonstrate the ability of targeted PTMs to alter binding specificity of organelle tethers and suggest a potential mechanism for the control of inter-organelle communication.

P1208/B212

INF2 Regulates Organelle Motility via ER-associated Actin.

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Charcot-Marie-Tooth (CMT) disease is the most common inherited neurological disorder, affecting ~1 in 2500 people. It is a peripheral, length-dependent neuropathy defined by progressive degeneration of the nerves in the distal parts of the body. Mutations in a wide variety of proteins cause CMT. Many of these proteins play a direct or indirect role in organelle mobility. Given this fact and the fact that cells affected in CMT (peripheral neurons) are the longest cells in the body, it is hypothesized that CMT is a result of impaired organelle mobility. INF2 is one of the proteins mutated in CMT. It is an ER-anchored formin protein which promotes actin assembly and modulates mitochondrial morphology and motility through this actin polymerizing activity. All CMT-causing INF2 mutations result in a constitutively active form of INF2. We predicted that this aberrant activity causes excess accumulation of ER-associated actin around mitochondria, restricting their mobility. We have demonstrated that mitochondrial mobility is reduced by exogenous expression of two CMT-causing INF2 mutations. We also studied the mobility of other organelles and found that expression of these mutants also reduces the mobility of endosomes and lysosomes, broadening the potential organelles that may play a role in the pathomechanism of CMT. We further validated these results in primary human fibroblasts from two CMT patients harboring endogenous INF2 mutations as well as neurons directly converted from these fibroblasts. Neurons derived from CMT patient fibroblasts also display neuronal swellings, consistent with a neurodegenerative phenotype. The reduction in organelle mobility is reversible by pharmacologically depolymerizing actin, supporting the model of excessive actin polymerization limiting organelle movement. To avoid the toxic side-effects of wholesale actin depolymerization, we developed a novel tool to specifically disrupt ER-associated actin. We found that this tool results in rescue of organelle mobility without causing obvious toxicity and reverses the neuronal swellings observed in derived CMT neurons. These findings demonstrate a potential mechanism by which INF2 mutations lead to excessive ER-associated actin, impaired organellar mobility, and ultimately, peripheral neuron degeneration.

P1209/B213

Symmetry Breaking by Polarizing ER-PM Contact Sites.

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Directed cell migration is driven by the front-back polarization of intracellular signalling. Receptor tyrosine kinases and other inputs activate local signals that trigger membrane protrusions at the front. Equally important is a long-range inhibitory mechanism that suppresses signalling at the back to prevent the formation of multiple fronts. However, the identity of this mechanism is unknown. Here we report that endoplasmic reticulum-plasma membrane (ER-PM) contact sites are polarized in single and collectively migrating cells. The increased density of these ER-PM contacts at the back provides the ER-resident PTP1B phosphatase more access to PM substrates, which confines receptor signalling to the front and directs cell migration. Polarization of the ER-PM contacts is due to microtubule-regulated polarization of the ER, with more RTN4-rich curved ER at the front and more CLIMP63-rich flattened ER

at the back. The resulting ER curvature gradient leads to small and unstable ER-PM contacts only at the front. These contacts flow backwards and grow to large and stable contacts at the back to form the front-back ER-PM contact gradient. Together, our study suggests that the structural polarity mediated by ER-PM contact gradients polarizes cell signalling, directs cell migration and prolongs cell migration.

P1210/B214

Keep Calmin and Carry On: Tethering the ER to F-actin Enables Cell Migration.

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The endoplasmic reticulum (ER) is an interconnected organelle characterized by a variety of membrane shapes, including sheets and tubules. Interactions between the ER and microtubule cytoskeleton are well-characterized, but an unresolved question is how the ER interacts with the actin cytoskeleton, which largely governs cell shape and movement. Using proximity-labeling spatial proteomics, here we identify a new ER tubule-specific protein, calmin (CLMN), and propose it mediates ER-actin tethering and regulates cell migration. Mechanistically, CLMN contains F-actin-binding calponin homology (CH) domains related to the CH domains of nesprin proteins. Live and fixed-cell imaging of overexpressed and endogenously-tagged CLMN reveals that CLMN localizes to ER tubules and basolateral punctae that are positive for actin and focal adhesion markers. 3D Structured Illumination Microscopy (3D-SIM) reveals that CLMN-positive ER innervates a sub-population of focal adhesions. Structure-function analysis reveals that the interaction between the CH1 and CH2 domains tunes the extent and specificity for CLMN localizing to F-actin, while a C-terminal transmembrane domain mediates its ER localization. Thus, we propose that CLMN is a new ER-to-actin tether. Using imaging-based and functional assays, we show that CLMN is required for proper actin organization. At the same time, CLMN-deficient cells display slowed migration and have more adhesions, indicating that CLMN tethering of ER to actin may mediate adhesion turnover. Live imaging of calcium biosensors shows that loss of CLMN impacts calcium signaling at the leading edge, suggesting CLMN influences local calcium signaling through regulating ER-actin contacts. Collectively, we propose that CLMN tethering of the ER to actin associated with focal adhesions promotes calcium dynamics to enable adhesion disassembly, thereby ensuring proper cell migration. This work identifies a new ER tubule-localizing protein, reveals the first demonstration of an ER-actin molecular tether, and highlights how organelle-cytoskeleton interactions play a significant role in cell motility.

P1211/B215

Investigating alterations in mitochondrial morphology and membrane contact sites following acute lipid depletion.

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Effective inter-organelle communication is essential for the ability of a cell to adapt, respond, and thrive in dynamic environments. Membrane Contact Sites (MCSs) play a crucial role in this process, acting as molecular bridges that physically connect organelles, ensuring their proper distribution and facilitating the transfer of essential biomolecules. Despite their importance in cellular organization, the mechanisms driving MCS formation remain poorly understood. Our study focuses on how phospholipid composition influences MCS formation and organelle morphology. We engineered strains to alter the synthesis pathways of the most abundant cellular phospholipids, phosphatidylethanolamine (PE) and

phosphatidylcholine (PC). Given that mitochondria interact with almost all organelles via MCSs, we used mitochondria as our model organelle. To overcome the limitations of traditional gene knockouts—in which intermediate states are often missed due to compensatory mechanisms—we employed an auxin-inducible protein degradation system to acutely deplete lipid-synthesizing enzymes in the Kennedy Pathway. This approach allowed us to capture alterations in MCSs and organelle morphology that would otherwise be overlooked when examining the terminal phenotypes observed in gene knockouts. Our findings reveal that disruptions in PE and PC synthesis pathways significantly alter mitochondrial morphology, resulting in fragmented, "beads-on-a-string," and collapsed structures—phenotypes that were previously missed in conventional gene knockouts of Kennedy Pathway enzymes. Remarkably, these defects partially revert to a near wild-type state within 90 minutes of protein depletion, suggesting the activation of a compensatory mechanism. Future work will focus on elucidating the role of MCS regulation in restoring normal mitochondrial morphology. By expanding our understanding of MCS function from a membrane-centric perspective, this study aims to uncover the pivotal role of lipids in coordinating organelle communication and cellular organization.

P1212/B216

Disruption of Lipid droplet and Mitochondria Communication in a Rab8a-dependent Way Contributes to Hypoxia Reoxygenation Injury in Cardiomyocyte.

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Lipid droplet tethering with mitochondria to facilitate fatty acid oxidation is important for cardiomyocytes to counteract hypoxia reoxygenation stress. We demonstrated that hypoxia reoxygenation in cells or myocardial ischemia reperfusion in mice reduced expression and phosphorylation of Rab8a, a mitochondrial receptor forming the tethering complex with the lipid droplet-associated perilipin 5 in cardiomyocytes. Indeed, long chain fatty acid mobilization to mitochondria after hypoxia reoxygenation was decreased in Rab8a-knockout cells. To examine the fate of lipids stored in lipid droplets in H9c2 cells after hypoxia reoxygenation, we used a long chain fatty acid tracking assay and found reduced transfer to mitochondria. Nevertheless, lipid droplet content was reduced by hypoxia reoxygenation and we found this occurred via both lipophagy and lipolysis (neutral and acidic). Mitochondria fragmentation occurred upon hypoxia reoxygenation, but this process did not contribute to lipid droplet breakdown. Intervention with ALY688, an adiponectin receptor agonist, restored Rab8a expression and partially restored the lipid droplet and mitochondria tethering to reduce the metabolic stress. These findings identify new regulatory mechanisms underlying the beneficial effects of adiponectin mimetics in ischemic heart disease.

P1213/B217

The Molecular Mechanism of On Demand Sterol Biosynthesis at Organelle Contact Sites.

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Contact sites are specialized zones of proximity between membranes of two organelles that are essential for organelle communication and coordination. The formation of contact sites between the largest organelle in the cell, the Endoplasmic Reticulum (ER), and other organelles, relies on a unique membrane environment that is enriched in sterols. However, how these essential ER subdomains are formed and maintained had not been understood. We found that the yeast membrane protein Yet3, the homolog of human BAP31, is localized to multiple ER contact sites. We show that Yet3 interacts with all the enzymes that catalyze the post-squalene steps in the ergosterol biosynthesis pathway, and recruits them to specific membrane areas on the ER, creating sterol-rich domains. Increasing sterol levels at ER contact sites causes its depletion from the plasma membrane (PM) leading to a cellular compensatory reaction. We demonstrate that these changes dramatically alter cell metabolism, and affect the structure and function of major organelles. Taken together, our data places Yet3 as a master metabolic regulator, whose activity to provide on-demand sterols at contact sites shapes the metabolism of fats and amino acids, and balances respiration and trafficking. A molecular understanding of this protein's functions gives new insights into the role of BAP31 in development and pathology.

P1214/B218

Contact-FP: Employing dimerization-dependent fluorescent proteins to study organelle contact sites and lipid droplet biology.

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Eukaryotic cells are defined by their ability to compartmentalize biochemical processes into discrete membrane-bound organelles. While compartmentalizing chemically incompatible processes is critical, many organelles interact to coordinate functions. Membrane contact sites (MCSs), sites of close apposition between membrane-bound organelles, usually defined as being within 10-100 nm, facilitate these interactions and allow the exchange of ions, lipids and proteins between organelles. While MCSs have often been visualized through electron microscopy, this process is incompatible with live cell imaging. The diffraction limit of light in conventional confocal microscopy cannot distinguish between *bona fide* contact sites and close but not interacting organelles. To visualize dynamics of MCSs in live cells, we have developed a series of reversible dimerization-dependent fluorescent protein (ddFP) probes to visualize MCSs between membrane-bound organelles including the plasma membrane, endoplasmic reticulum (ER), mitochondria, peroxisomes, lysosomes, and lipid droplets (LDs). When the heterodimers of the probes, targeted to two organelles of interest, come into proximity at MCSs, the "A" monomer brightly fluoresces upon dimerization. We have demonstrated that these probes correctly localize to and can detect changes in LD-organelle MCS number and/or size under genetic and chemical perturbations, such as overexpression of tether proteins or changing nutrient levels. Our data suggest that by titrating the amount of ddFP transfected, we can minimally perturb or, at higher concentrations, induce MCSs between the organelles. We used ddFP expression to test the effect of modulating MCSs between LDs and other organelles on LD morphology and dynamics. Interestingly, our data suggest that inducing mitochondria-LD MCSs do not affect LD size, while increasing ER-LD MCSs increases LD size. LDs, dynamic phospholipid monolayer-bound organelles that originate from the ER store neutral lipids. Better understanding how MCSs between the ER and LD respond to stress and influence individual organelle dynamics may elucidate how these organelles coordinate the handling of lipid and proteins and control lipid metabolism under stress. Our Contact-FP toolkit represents a useful resource for cell biologists to understand the role and dynamics of MCSs, including ER-LD contact sites.

Mechanobiology in Cell Structure and Function 1

P1215/B220

αV-class integrins identify ECM-context to license cellular mechanics and mechanotransduction within seconds.

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The cellular ability to biophysically and biochemically recognize extracellular matrix proteins is fundamental to adhesion, mechanics, migration, and morphogenesis, and influences homeostasis and disease. However, the mechanisms underlying integrin-mediated mechanosensing of the arginine-glycine-aspartic acid (RGD)-motif of vitronectin and fibronectin remain elusive. Here, we discover that within seconds of sensing vitronectin, αV-class integrins initiate and strengthen adhesion biphasically through complementary mechanotransduction pathways, which rely on the catch bond behavior of single αVβ3 integrins. The first adhesion phase requires αVβ3 and αVβ5 integrin-associated actomyosin and FAK activity, while αVβ5 integrin additionally requires clathrin-mediated endocytosis. With elevating mechanical load, the second phase requires αVβ3 integrin-directed Arp2/3, cSrc, and PI3K signaling that dominates αVβ5 integrin in organizing the consensus adhesome on vitronectin. Simultaneously, αVβ5 integrin regulates the mechanical stiffening of fibroblasts. Thus, αV-class integrins exhibit rapid RGD-motif- and β-subunit-specific programs to synergistically guide mammalian cell adhesion and mechanics upon encountering diverse extracellular environments.

P1216/B221

Force Transmission via Dynamic Stretching of Talin as Revealed by Live-cell Single-molecule Imaging.

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Focal adhesions (FAs) are responsible for transmitting intracellular forces to the extracellular matrix (ECM). Talin is an essential FA protein that links actin filaments (F-actin) to integrins. F-actin constantly moves on FAs, yet how Talin simultaneously maintains the connection to F-actin and transmits forces to integrins remains unclear. To address this issue, we performed Single-Molecule Speckle (SiMS) microscopy which elucidates true functions and kinetics of individual Talin molecules in live cells. We recently revealed that the majority of Talin molecules are bound only to either the moving F-actin network or the substrate whereas a small portion of Talin is liked to both structures via elastic transient clutch (Yamashiro et al., Nat Commun, 14:8468, 2023). By reconstituting Talin knockdown cells with Talin chimeric mutants, we found that the stretchable property of the Talin rod domain, but not its binding to Vinculin, is critical for force transmission. Simulations suggest that unfolding of the Talin rod subdomains increases in the linkage duration and work at FAs. Our study elucidates a force transmission, in which stochastic molecular stretching bridges two cellular structures moving at different speeds.

P1217/B222

Defining the effects of fibrotic stiffening on airway epithelial phenotype.

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Epithelial cells that line the airways of the lungs are exposed to air on their apical surface and a pliable substratum on their basal surface. Culturing isolated epithelial cells on membranes at an air-liquid interface (ALI) mimics the apical microenvironment, but the membranes themselves are much stiffer than the mesenchymal tissues of the lung. To address this issue, we grafted the surfaces of transwell membranes with polyacrylamide (PA) gels with stiffnesses that covered the physiological range of the healthy and fibrotic lung. We used this novel experimental platform to test the effects of fibrotic stiffening on the phenotype of human bronchial epithelial cells. Specifically, we characterized their morphology and mechanical signaling by measuring projected cell area and nuclear localization of the mechanosensor YAP. We also measured transepithelial electrical resistance (TEER), conducted RT-PCR analysis for markers of differentiation, and performed immunostaining analysis for markers of tight junctions. Our data show human bronchial epithelial cells exhibit distinct morphologies and functional phenotypes when cultured at ALI on PA gels of different stiffness. On stiffer substrata, these cells show increased projected cell area and nuclear localization of YAP. We also found that the permeability of the epithelium is sensitive to substratum stiffness, which has implications for barrier function of the lung under conditions such as fibrosis. This engineered culture model can be used to investigate the effects of fibrotic stiffening on viral transmission and recovery after airway injury.

P1218/B223

IRE1 is Involved in Translation Control Under Mechanical Stress.

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Over the span of their life, cells in the body undergo a plethora of mechanical stresses. Proteins called mechanosensors sense mechanical stimuli and turn them into biochemical signals that activate downstream adaptation mechanisms. Since mechanobiological research is usually focused on the plasma membrane, the cytoskeleton and the nucleus, the endoplasmic reticulum has never been investigated in a mechanosensing context before. In our search for an ER-resident mechanosensor, we explored the mechanosensing potential of Inositol requiring protein 1 (IRE1), a major player in the unfolded protein response (UPR), that was also described to sense lipid bilayer stress in the ER.
Our data shows that confining cells to 3µm for 30min resulted in IRE1 phosphorylation. When testing the activity of the PERK cascade, we noticed that the PERK-target eIF2a was dephosphorylated, indicating inactivity of the pathway. Together with the absence of unfolded proteins, this suggests that confinement activates IRE1 independently of the UPR. Furthermore, mechano-activated IRE1 does not result in canonical UPR signaling (splicing of XBP1).
Since dephosphorylation of eIF2a suggests a change of translation levels, we investigated translation levels in confined cells. Strikingly, SUnSET translation assays show that translation under confinement decreases drastically when IRE1 is inhibited. Our data also shows that translation inhibition by cycloheximide treatment mimics IRE1 activation without XBP1 splicing, which indicates that inhibition of translation might constitute the trigger for IRE1 activation under confinement.
Finally, we explored potential reasons for translation inhibition under confinement. We hypothesized that confinement affects cytoplasmic crowding. To this end, we tracked the movement of fluorescent

Genetically Engineered Microparticles (GEMs) to calculate the diffusion coefficient. We found that confinement resulted in a decrease of cytoplasmic crowding, a condition that was previously associated with inhibition of translation.
In summary, we propose a model whereby confinement inhibits translation by decreasing cytoplasmic crowding. This in turn results in IRE1 activation, which serves to maintain physiological translation levels. Our results establish IRE1 as an ER-based mechanosensor and highlight an unprecedented role in the control of cellular proteostasis.

P1219/B224

Ultrafast contraction in the giant cell *Spirostomum* is driven by fishnet of Ca2+-triggered centrin-Sfi1 mesh.

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Spirostomum is a large unicellular ciliate capable of contracting its nearly millimeter long body to a quarter of its length in less than five milliseconds. Measured as fractional shortening, this motion is an order of magnitude faster than actomyosin-based contraction. Powering this contraction are myonemes, cortical protein networks, often rich in centrin and Sfi1 homologs, that are found in many protists. Fast contraction, slow elongation, and calcium-triggering are hallmarks of myoneme-based motion, but its biochemical basis and molecular mechanism are not well understood. To probe their role in Spirostomum contraction, we measure changes in cortical structures, including centrin/Sfi1, microtubules, and the plasma membrane, via immunofluorescence and electron microscopy. We use these experimental results to inform models of the whole cell and of underlying protein complexes. We show evidence from both experiment and modeling that calcium-triggered reorganization of centrin/Sfi1 is responsible for contraction. We recapitulate organismal-scale contraction in mesh simulation experiments and demonstrate the importance of the fishnet-like structure of the myoneme for reproducing experimentally observed changes in cell shape. We provide further evidence that unlike molecular-motor based motion that directly couples ATP hydrolysis to mechanical work, myoneme contraction may instead be powered by an entropic spring whose properties are modulated by binding to calcium. Together, these results provide a more cohesive, multiscale model for the contraction of *Spirostomum*. Deeper understanding of how single cells can execute extreme shape changes holds potential for advancing cell biophysics, synthetically engineered contractile machinery, and biologically inspired robotics.

P1220/B225

Emergent mechanical properties of growing epithelial monolayers on soft 2D surfaces.

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Epithelial morphogenesis during development, including tissue shaping and patterning, occurs under a wide range of physicochemical conditions. However, most studies of tissue mechanics in epithelial morphogenesis have been conducted on rigid, glass surfaces, limiting our understanding of tissue growth on substrates of physiological stiffness. Therefore, the goal of this study is to establish a two-dimensional epithelial monolayer model simulating physiologically relevant conditions to elucidate the

mechanisms of cell-cell and cell-ECM interfaces, including the actomyosin network.
Here, we used soft polyacrylamide (PAA) gels that replicate the stiffness of ECM, upon which polarized MDCK monolayers exhibiting either normal or aberrant plasma membrane and actin cytoskeleton integrity were grown. We utilized I-BAR-containing IRSp53 depleted in MDCK cells to determine changes in apical surface mechanical properties using high spatiotemporal resolution Atomic Force Microscopy nanomechanical mapping. From the obtained AFM maps, we measured changes in the mechanical properties of the cellular actin cortex and cell-cell junctions. We observed that knockout of IRSP53 in MDCK cells plated on glass and 8kPa PAA gels significantly reduces the apical surface mechanical properties. While the reduction is more profound for cells grown on glass, this observation reinforces the significance of incorporating physiological relevance in mechanobiology. Therefore, our results indicate that the mechanical attributes of polarized epithelial monolayers are, in part, dependent upon the mechanics of their substrate and that IRSp53 acts as a plasma membrane and cell-cell junctions mechanosensor.

P1221/B226

The mechanics and cell biology of apical bulkheads in the bile canaliculi of the liver.

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The bile canaliculi are a set of thin but highly interconnected tubes which carry bile away from the hepatocytes which produce it to the larger bile ducts which transport it out of the liver. Bile canaliculi lumina are formed between just two cells, making them unique and mechanically interesting cellular structures. Despite their crucial physiological role, the small diameter of these lumina (only a few microns) has made research into their spatial structure and dynamics difficult. However, significant progress has been made in the last few years on understanding the mechanical basis of their formation during development. One such contribution was the description of “apical bulkheads”: shield-like protrusions into the bile canaliculus lumen which connect the apical surfaces of the two hepatocytes, but do not seal the lumen off [1]. Following this work, we sought the physiological role of apical bulkheads, which we found are prevalent both during liver development in mice and in diseased human livers. Using a combination of high-resolution imaging, laser ablation, and mechanical modeling, we found that apical bulkheads increase the pressure which bile canaliculi hold [2]. Imaging showed that structurally, bile canaliculi are surrounded by contractile actomyosin cortex, which extends into the apical bulkheads. Tight and adherens junctions seal and support the cell-cell interface, and also extend into the apical bulkheads. We included these structural features into a mechanical model of the bile canaliculus and apical bulkheads, implemented as a set of elastic surfaces in 3D space to capture the mechanical response at short timescales. Imaging and laser ablation data enabled us to parameterize the model, which we then used to find that apical bulkheads double the pressure the bile canaliculus holds for at a given diameter [2]. While these results suggest a function for apical bulkheads, they do not reveal the mechanism of their formation. New imaging data shows apical bulkheads are dynamic, extending into the lumen and retracting back on a timescale longer than minutes but shorter than hours. A minimal fluid model for the mechanics of apical bulkheads suggests they may form by a mechanical instability.

[1] Belicova, L. *et al.* Anisotropic expansion of hepatocyte lumina enforced by apical bulkheads. *Journal of Cell Biology* 220, e202103003 (2021).

[2] Bebelman, M. P., Bovyn, M. J. *et al*. Hepatocyte apical bulkheads provide a mechanical means to oppose bile pressure. *Journal of Cell Biology* 222, e202208002 (2023).

P1222/B227

Chemical Compensation to Mechanical Loss via PI3K/Akt-NHE1 Crosstalk.

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Mammalian cells sense and respond to physical stimuli through a complex system that integrates chemical and mechanical signals. The transduction of mechanical cues into chemical reactions modulates overall cell physiology, which, in turn, influences cell mechanics, allowing cells to adapt to their physical microenvironments. Understanding how the chemical and mechanical regulatory modules interact is crucial for elucidating mechanisms of mechanosensation and cellular homeostasis. In our study, we found that cells exhibit non-monotonic changes in migration, cell volume, and intracellular pH when subjected to physical stimuli and varying degrees of actomyosin cytoskeleton disruption. We discovered these non-monotonic responses are mediated by a chemical compensation mechanism, where the attenuation of cell mechanics stimulates the activity of PI3K/Akt pathway. This hyperactivation subsequently activates NHE1, thereby elevating intracellular pH, cell volume, and cell migration. Furthermore, we identified a competitive interaction between the PI3K/Akt and MAPK/ERK pathways—the two major regulators of cell proliferation and motility. This competition modulates the chemical compensation based on the relative activities of these pathways. We further discovered that NHE1 activity negatively feedbacks to regulate PI3K/Akt activity. Our mathematical modeling suggests that the negative feedback loops between PI3K/Akt and NHE1, as well as between actomyosin and PI3K/Akt, are essential for establishing the homeostatic state. Interestingly, this regulatory system is absent in certain cancer cell lines, such as HT1080 fibrosarcoma, highlighting a potential mechanistic divergence in different cell types. Our work reveals a critical compensatory mechanism between chemical and mechanical signals, providing novel insights into mechanosenation and the establishment of cellular homeostasis.

P1223/B228

Multimodal Correlative Confocal and SEM Imaging of Bone Exposed to Microgravity Reveals Disrupted Osteogenesis and Coupled Osteoclastic Osteolysis in *Cdkn1a* $^{-/-}$ Mice.

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Mechanical unloading of mouse bone in microgravity models disuse of weight-bearing bones on Earth and is associated with elevated CDKN1A expression and decreased mineralization. Conversely, deletion of *Cdkn1a* in bone marrow stromal osteoprogenitors significantly increases *in-vitro* osteoblastic proliferation and mineralized nodule formation, with enhanced osteogenesis in response to mechanical loading by uni-axial stretch. In this work, we sought to study this phenomenon *ex-vivo*, using microCT and multimodal correlative imaging via confocal microscopy and scanning electron microscopy (SEM).

We investigated cellular mechanisms of osteogenesis and osteoclastic bone degradation in the femur of 16-week-old female B6129SF2/J wildtype (WT) and *Cdkn1a* $^{-/-}$ (KO) mice flown in microgravity for 30 days. Confocal imaging of the optically cleared femoral endosteum, stained with two calcein injections on-orbit five days apart, revealed an increased population of osteoblast progenitors in microgravity. Additionally, the surface area of multicellular osteocytic mineralization surfaces was reduced compared to ground controls, indicating a disruption of osteogenesis and bone maintenance. The KO demonstrated increased magnitude of this pro-osteogenic effect in normal loading but showed no sensitivity to microgravity unloading. Same-sample correlative SEM imaging visualized areas of bone resorption by osteoclasts, which was increased by 28% in microgravity and by 64% in KO samples compared to the WT control. These results, together with greater progenitor osteogenesis, suggest osteoclastic degenerative activity is coupled to increased osteoprogenitor recruitment to the bone surface. MicroCT analysis of WT femurs from microgravity shows a 5% significant decrease in bone mass and cortical thinning, while KO mice have a much larger significant tissue loss, including 12% thinner cortical bone and 10% lower percent cortical bone area. This study suggests that CDKN1A plays a key role in the mechanosensitive negative regulation of osteoprogenitor proliferation and differentiation plus coupled osteoclastic differentiation, which may prevent excessive tissue turnover. Finally, this study demonstrates a novel correlative approach to quantifying multiple bone osteogenic parameters using confocal imaging of cleared mineralized tissue, SEM, and microCT of the same sample.

P1224/B229

Single-Cell mRNAseq Profiling and Correlative Imaging of the Femoral Bone Marrow Endosteum in Mice Reveals Increased Remodeling of the Osteoimmune Microenvironment in Microgravity.

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Microgravity is a model for mechanical unloading, replicating disuse of weight-bearing bones on Earth, and is associated with increased immune activation and disrupted bone remodeling. This study investigates the role of immune-osteal interactions in femoral bone remodeling under microgravity. T-helper cells and macrophages, that play roles in regulating osteogenesis and osteoclastogenesis, were analyzed using single-cell transcriptomics (n = 3/group) of 20-week-old female B6129SF2/J mouse femurs exposed to 30 days of microgravity. Subtypes of common bone marrow cell populations were identified using cell surface and intracellular markers and evaluated using informed t-SNE clustering. Results show that Th1 and Th2 cells, which support osteogenesis, exhibit increased populations and higher expression of IFN-γ and IL-4, anti-osteoclastogenic cytokines, compared to ground controls. In addition, M1 pro-inflammatory macrophages, which promote osteoclastogenesis, are also more abundant in microgravity, with elevated expression of TNF-α, IL-1β, and IL-6 inflammatory cytokines. Furthermore, osteal macrophages often associated with canopies, over regions of bone remodeling, also show increased numbers in the marrow with upregulation of TNF-α and IL-1β, indicating a shift towards a pro-inflammatory M1 phenotype, that can contribute to degenerative bone remodeling. Conversely, numbers of regulatory B and T cells that maintain immune homeostasis and prevent excessive inflammation are reduced in microgravity. Regulatory T cells also show decreased expression of CTLA-4 and IL-10, while regulatory B cells exhibit elevated TGF-β expression, which may promote

osteoclastogenesis in the context of increased inflammatory cytokines. Correlative imaging techniques, including calcein labeling of mineralizing cells and scanning electron microscopy, show significant bone remodeling uncoupling, reducing osteogenesis, and increased osteoclastic activity on the femoral endosteal surface under microgravity. These findings suggest that microgravity induces a shift towards a pro-inflammatory, pro-osteoclastogenic environment, exacerbating bone turnover and uncoupling of bone formation and resorption, analogous to disuse osteopenia observed on Earth.

P1225/B230

Enhanced Nanoscale Viscoelastic Properties and Force Generation of T Lymphocytes During Immune Synapse Formation.

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The cell-cell interface between a T-cell and an antigen-presenting cell, widely known as the immunological synapse (IS), is critical for T-cell activation, driven by the T-cell receptor (TCR) binding to its specific peptide antigen. Several studies have shown that mechanical properties of T-cells during activation play an important role in many cellular functions such as proliferation, migration, and cytotoxic activity. The formation and maintenance of the IS are supported by force generation through the dynamic interaction of actomyosin and microtubule cytoskeletal networks. However, the way this force generation influences the mechanical properties of T-cells is not yet fully understood. Here, we use high spatiotemporal resolution Atomic Force Microscopy to quantify the viscoelastic response via common mechanical parameters, namely the storage modulus, loss modulus, and loss angle across multiple timescales at the nanometer length scale. In addition, we used Traction Force Microscopy to quantify the traction stresses generated by T-cells on soft silicone gels during IS formation. Our findings show that T cells display structurally diverse viscoelastic properties at the nanoscale level during IS formation induced by CD3/CD28/LFA-1 co-stimulation. Particularly, we observe significantly higher elastic and viscous properties in the edge and central regions, while the peripheral transition region is softer and more fluid. These results align with changes in the actomyosin cytoskeleton architecture. Moreover, our results show that cytoskeletal perturbations of different filamentous actin regulatory proteins in T-cells lead to changes in elasticity and fluidity of T-cells, as well as changes in traction stresses generated by T-cells during IS formation. Therefore, determining the relationships between key cytoskeletal structures at the IS and the cell's local and global mechanical properties is crucial for the maintenance and formation of IS, offering a deeper understanding of T-cell activation and the actomyosin cytoskeleton dynamics.

P1226/B231

Assembly of the surface layer in the thermoacidophilic archaeon *Sulfolobus acidocaldarius* and its role in cell division.

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Recent work has uncovered that many of the cellular machines that govern core biological processes in eukaryotes have their origins in archaea. This includes the ESCRT-III machinery, which plays a conserved role in membrane remodelling during cell division in both archaea and eukaryotic cells. At the same time, the mechanism of archaeal cell division is also likely influenced by the unique aspects of archaeal

cell biology: the archaeal lipid membrane and the highly glycosylated protein surface layer (S-layer) that envelopes most archaeal cells.
Using the thermoacidophilic archaeon *Sulfolobus acidocaldarius* as a model system in which to explore the role of the S-layer, we investigate its assembly and function in detail. We show that SlaA self-assembles to form an S-layer lattice which extends through the addition of monomers at its margins. The lattice is attached to the cell surface by a novel Thermopsin-like membrane protein along with its canonical membrane anchor SlaB.
Using a panel of S-layer mutants, we further identify conditions under which the S-layer lattice is preferentially recruited to the division neck. Live cell imaging performed at high temperatures revealed changes in cell morphology during division and high rates of division failure in S-layer mutants. Taken together, these results reveal the rules of S-layer self-assembly and show how this surface layers aids the control of cell shape and the execution of cell division in an archaeon.

P1227/B232

ZO-2 modulates JAM-A and γ-actin junctional recruitment, apical membrane and tight junction tension, and cell response to substrate stiffness and topography.

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This work aimed to test the importance of the tight junction (TJ) protein Zonula occludens-2 (ZO-2) for regulating mechanical forces in epithelial cells. For this purpose, we measured the rigidity of the apical membrane in ZO-2 KD epithelial MDCK cells by indentation with an atomic force microscope. We observed a reduced rigidity in comparison to parental MDCK cells. The diminished apical rigidity observed correlated with the instability and loss of the ring of microtubules associated with the TJ and a reduced gamma-actin concentration at the TJ belt. Instead, we observed increased tension at bicellular TJs measured by FRET with a ZO-1 tension probe and at tricellular TJs determined by the junctional recruitment of vinculin. In contrast, tension at the adherens junction determined with an E-cadherin probe was unaltered by the absence of ZO-2. The lack of ZO-2 triggered a diminished association of JAM-A to the cell borders, facilitating the junctional accumulation of p114RhoGEF and afadin that enhanced tension at the TJ and initiated the formation of basal stress fibers. The increase in TJ tension triggered by the lack of ZO-2 has a deleterious effect on cell-cell adhesion, as monolayer integrity is lost due to the appearance of holes when the cells are plated on hydrogels with a rigidity of 20 kPa, like that found by epithelial renal cells in vivo. Through in silico analysis, we characterized the molecular-level interactions of the ZO-2 PDZ-2 domain and JAM-A tail and computed the binding energy that leads to stable protein-protein association. The response of the cells to the rigidity and topography of the substrate was also affected by the lack of ZO-2. Thus, ZO-2 KD cells retarded their elongation and formation of aggregates when plated in hydrogels covered with collagen IV or fibronectin and increased the internalization of claudin-4 and the nuclear accumulation of Snail and YAP when plated in nanostructured ridge arrays.

P1228/B233

The Age-Associated Increase In Ovarian Stiffness Impairs Follicle Development And Oocyte Quality Through Early Modulation Of Follicles' Transcriptome.

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Oocyte quantity and quality are severely compromised with female reproductive aging. We previously reported that with aging the mouse ovary becomes stiffer. However, the role of stiffness on ovarian function and oocyte quality is unknown. This study aims to use an alginate-encapsulated *in vitro* follicle culture that mimics age-associated changes in ovarian microenvironment to identify whether stiffness is a novel mechanism mediating folliculogenesis and oocyte quality. We synthesized hydrogel recapitulating the soft (0.5%, 1.79±0.08kPa) and stiff (2%, 4.56±2.03kPa) environments. Secondary follicles from CD1 mice (N=3 replicates) were cultured in those hydrogels for up to 12 days. Follicles cultured in stiff environment showed significant reduction in follicle size compared to follicles in soft environment (0.5% 226.9±17.4um, 2% 160.8±9.9um,p< 0.0001). These differences were triggered by granulosa layers since no changes were detected in oocyte size (oocytes: 0.5% 66.19±5.7um, 2% 60.6±4.5um,p=0.401; granulosa cells: 0.5% 160.04±13.4um, 2% 103.37±16.5um,p<0.0001). To explore if granulosa cells are not proliferating in a stiff environment, we assessed estradiol synthesis and found that estradiol levels were reduced in 2% (0.5% 11.3±15.9ng/ml, 2% 0.3±0.5ng/ml,p=0.296), thus stiff environment impact granulosa cell viability. We then evaluated if stiff environments impact gamete quality. Oocyte quality significantly declined in follicles cultured in 2% hydrogels, with 68.9±16.8% of oocytes degenerated, compared to 23.6±9.2% in 0.5%. Since the effects of stiffness on follicle development were already evident at D2, we investigated early-changes in follicles' transcriptome. 120 secondary follicles were cultured in 0.5% or 2%, and analyzed by RNAseq at 3h, 6h, 12h and 24h. We analyzed follicles' gene expression at each timepoint comparing 0.5% and 2%. We found few differentially expressed genes (DEG) at 3h, a peak of DEG at 6h, followed by a decrease which reached a plateau at 12h and 24h. DEGs at 6h were involved in response to external stimuli, inflammation and apoptosis, suggesting that at 6h follicles' transcriptome is more sensitive to changes in ovarian biomechanics. We then analyzed DEGs in a time-dependent manner. We identified 1029 DEGs in follicles cultured in 2% at 24h compared to 3h. Most of the upregulated genes were associated with extracellular matrix (ECM) remodeling, collagen, and connective tissue development. In the 0.5% condition, 1282 DEGs were identified, with upregulation of genes related to metabolism and cell cycle at 24h. Overall, we demonstrated that age-associated ovarian stiffness is a novel regulator of folliculogenesis. Work supported by NIH-K99/R00-HD108424 to FAR.

P1229/B234

Cell size reduction and solid stress accumulation in proliferating multicellular spheroids.

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To maintain proper function, cells must regulate a consistent distribution of sizes, a process known as cell size homeostasis. Achieving cell size homeostasis requires precise coordination between growth and division. In mammalian cells, growth occurs under various mechanical conditions. For instance, during proliferation in solid tumors, cells exert pressure on their neighbors and the surrounding extracellular matrix (ECM), resulting in the buildup of solid stress within the tumors. How does this solid stress affect cell proliferation and cell size homeostasis? To explore this, we utilize a multicellular cancer spheroid

model to simulate solid tumor development, with spheroids cultured in hydrogels with tunable stiffness and viscoelasticity to vary confinement. We quantified individual cell size in proliferating spheroids using both in situ 3D segmentation from confocal microscopy and spheroid dissociation followed by flow-based particle sizing (Coulter counter). We measured stress accumulation within the spheroid by incorporating elastic beads and tracking their deformation over time. Our findings indicate that as the spheroids proliferate, cell size decreases while solid stress increases over time. These results suggest that mechanical stress can disrupt cell size homeostasis.

P1230/B235

Imbalanced Proteome Underlies a Hypo-Osmotic Like Stress in Aneuploid Cells.

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Aneuploidy, a condition in which cells harbor an abnormal number of chromosomes, is a hallmark of genome instability and is present in approximately 90% of solid tumors and 70% of hematopoietic malignancies. This chromosomal imbalance leads to proteome imbalance, disrupting the stoichiometric balance of proteins and triggering an intracellular hyper-osmotic pressure and a general hypo-osmotic-like stress to the whole cell. As a result, cells experience abnormal swelling and a breakdown in the proportional relationship between cell size and ploidy.Previous studies have attributed this disproportionate cell growth to basic thermodynamic models, yet the precise biophysical mechanisms linking proteome imbalance to these cellular defects remain unclear. To further investigate these mechanisms, we employ Fluorescent Exclusion Measurement to analyze cell volume dynamics in response to hypo- and hyper-osmotic shocks. Our experiments suggest that proteins influence cell size through a dry volume and counterion mechanism. Based on these findings, we developed a new biophysical model, integrating insights from the classic Pump and Leak Model, to explain the out-of-scaling relationship between protein levels and cell size.Finally, we propose that cells regulate their size through osmotic-ploidy scaling, governed by biophysical principles underlying proteome scaling. These insights may shed light on the role of proteome dysregulation in cancer progression.

P1231/B236

Glycocalyx mediated regulation of cancer cell adhesion, invasion and survival.

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Glycocalyx encompasses the pericellular polymers that are extensively glycosylated and its overexpression is associated with cancer progression and metastasis. To further elucidate the mechanisms by which the glycocalyx enhances cell survival and invasion, we studied breast cancer patient samples and cell lines heterogenous in Mucin-1 (MUC1 - major glycoprotein upregulated in breast cancer) and glycan expression. We observed sub-type specific expression of MUC1 in patient tumours, however our 2D motility and 3D invasion experiments on differentially expressing MUC1 cell lines revealed a dependence of cell motility on the presence of bulky glycocalyx. Similarly, MUC1-based sorted sub-populations of MCF-7 cell line revealed fastest motility and invasiveness in intermediate

MUC1-expressing cells, with glycocalyx disruption abolishing these effects. Upon closer investigation, we observed that MUC1 modulates its effects by differentially regulating cell-substrate adhesion in a substrate-dependent manner. In contrast to inducing cell rounding on collagen-coated substrates, high MUC1 level promoted cell adhesion and conferred resistance to shear flow on substrates coated with the endothelial surface protein E-selectin. As cells pass through confining micro-environments during metastasis and come across cellular stresses of the magnitude that may lead to cell death; we therefore next set-up transwell migration through 3 µm pores to study cell survival. We isolated two distinct sub-populations from the MDA-MB-231 cell line and observed that the early migrating cell sub-population possesses a bulkier glycocalyx and undergoes lesser DNA damage and nuclear rupture. Interestingly, enzymatic removal of glycocalyx led to disintegration of the nuclear membrane indicated by increased cytoplasmic localisation of Ku70/80, increased nuclear blebbing and reduction in nuclear area. Together, our results provide mechanistic view of glycocalyx mediated cell adhesion, invasion and survival.

P1232/B237

Bioinformatic analysis of calcium-triggered contractile systems in Spirostomum and related ciliated protozoa.

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Some ciliated protozoa use cytoskeletal motors that do not require ATP to generate force. These motors, triggered by calcium, drive contractile processes, some of which are ultrafast. The molecular basis of ultrafast contractility in ciliates needs to be better understood. We used proteomic analysis of cytoskeletal preparations of *Spirostomum* to initiate the identification of the critical proteins. Prior studies had suggested that the contractile myoneme of Spirostomum and other heterotrophs were built from a centrin-like calcium-binding protein and an Sfi1-like repeat protein. The proteomic analysis discovered several centrins in the derived proteome of *Spirostomum semivirescens*. A candidate for the Sfi1-like protein was identified by BLAST searches using the *Paramecium* giant centrin-binding proteins as queries. The initial hit was seen to contain tandem repeats of a 69 amino acid sequence. Searches of the *Spirostomum* proteome identified multiple proteins containing variable repeat numbers. Independently, nanopore sequencing of Spirostomum minus (*Sci Adv.* 2023 Feb 22;9(8):eadd6550) revealed two giant proteins containing many such Sfi1 repeats. We have analyzed Spirostomum centrin and Sfi1 repeat proteins from *Spirostomum minus,* several other *Spirostomum* species and their homologs in heterotrich ciliates species such as *Stentor* and *Blepharisma*. From this analysis, we have identified critical aspects of the architecture of these proteins, which have provided insights into possible mechanisms of intracellular assembly and ultrafast contraction of myonemes. While homologs of these proteins are present in other groups of ciliates, their Sfi1-like repeats are much less regular compared to the giant centrin-binding proteins found in *Spirostomum*. These data have guided the design of a simplified system that reconstitutes these contractile assemblies in vitro, which are used for biophysical studies and structural analysis by cryoelectron microscopy.

P1233/B238

A Computational Model of *Drosophila* Furrow Invagination During Cellularization.

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The creation of an epithelial sheet transforms *Drosophila* embryos from a single cell directly into a tissue. To do this, apical, microvillus membrane is pulled between each nucleus in a process known as furrow invagination. Experimental measurements of furrow invagination velocities have shown that each new cell simultaneously undergoes slow-to-fast and fast-to-stalled transitions. Such changes can be a result of multiple intersecting molecular mechanisms, including, motor proteins, microtubules, and F-actin. In this work, furrow invagination is represented with a continuum model of a membrane-cortex composite, that considers the roles of cytoplasmic drag, motor protein forces, and membrane tension. We find that the viscous flow of cortical, transmembrane proteins through the lipid bilayer dictates furrow velocity. We propose that the slow phase is slow because there is a high density of microvilli, which increases cortical-membrane contact points, and results in higher viscous stress. We predict that the fast phase may benefit from fewer flowing, transmembrane proteins, thus reducing viscous stresses and promoting the slow-to-fast switch. Then, we replicate furrow stall from loss-of-function experiments, to show that membrane reservoirs are vital to a successful invagination. This work demonstrates how coupling between the cortex, the membrane, and microvillus- and exocytic-reservoirs affect the plasticity and timing of cell shape change.

P1234/B239

Mechanotransduction at The Golgi Apparatus - Understanding The Effects of External Cues and Internal Forces on Intracellular Trafficking and Secretion Kinetics.

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Cells can sense and respond to external forces and mechanotransduction events appear to be critical for most cellular functions. While mechanotransduction has been extensively studied at the plasma membrane and at the nucleus, the impact of forces on other organelles is still poorly known. Our project focuses on studying mechanotransduction at the Golgi apparatus (GA), a central organelle regulating intracellular transport pathways. We aim to answer the following questions: 1) Can external and internal forces propagate to the GA and impact its tension? 2) How is the tension of the GA regulated? 3) Do post-Golgi trafficking and polarized secretion depend on the tension of the GA?
To achieve these aims, we use a specific experimental set-up. 1) We apply and measure internal forces directly on the GA by manipulating a bead with optical tweezers. At the intracellular level, we have previously measured the mechanical properties of the GA using optical tweezers. We have also shown that internal forces perturb post-Golgi trafficking by delaying membrane fission of transport carriers and inducing the formation of long membrane tubules (Guet et al., 2014). 2) We monitor the effects on tension at the GA using fluorescent HaloFlipper probes; Haloflippers are fluorescent reporters of membrane tension and lipid order in intracellular membranes (Strakova K et al., 2020). 3) We apply external constraints by modulating substrate stiffness and follow post-Golgi trafficking of synchronously secreted cargoes using the RUSH assay (Boncompain G et al., 2012).
Our findings suggest that the GA is mechanosensitive and can modulate its membrane tension in response to external or internal constraints. Cytoskeletal protein disruption and modifications effectively modulate Golgi membrane tension. Furthermore, we have shown that external constraints

such as substrate stiffness has a strong impact on the function of the GA as it affects the trafficking kinetics of some selected cargoes. Our results should provide new fundamental insights in the role played by mechanical tension in force transduction at the level of the GA.

P1235/B240

How does macromolecular crowding limit protein production under pressure?.

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Cells growing in confined spaces eventually build up mechanical compressive stress. Growth-induced pressure (GIP) decreases cell proliferation in all kingdoms of living. In the case of the yeast *Saccharomyces cerevisiae*, we showed that proliferation decay is most likely due to a slowdown in protein production, concomitant to an increase in macromolecular crowding, both nuclear and cytosolic (Alric, Nat Phys, 2022). We hypothesized that increased intracellular macromolecular crowding could directly induce this decrease. However, we barely know about which steps of protein biosynthesis are limited by crowding, and if major pathways such as mTORC1 are downregulated under pressure. Here, we investigate the physical origins of macromolecular crowding and its effects on protein synthesis of the yeast *Saccharomyces cerevisiae*. All quantifications are conducted by optical approaches, imaging cells that grow within an elastic PDMS confining microfluidic chamber. We first showed, using quantitative phase microscopy, that the amount of macromolecules, including proteins, increased linearly with the pressure level using quantitative phase imaging. At a pressure level of 0.5 MPa, the dry mass of the cells increased by 50%, indicating the physical origin of the crowding is related to at least the accumulation of macromolecules. However, metabolic labeling of every newly synthesizing RNA with 5-ethynyl uridine (5-EU) showed that the mean transcriptional rate of the whole transcriptome was decreased by 7 times under 0.8 MPa of GIP. We next examined the dynamics of transcription of an individual gene via single-molecule live cell imaging. The transcription dynamics of GAL10 that were visualized by the introduction of PP7 repeats in the 5'UTR showed that the bursting pattern was modified by pressure. The average ON and OFF times of bursts, which represent the transcriptional state, are strongly dependent on pressure, while the elongation speed of RNA polymerase II and the frequency of the bursts were relatively constant. These results showed that the dwell time of transcription factors (Gal4 in the case of GAL10) was reduced, so we infer that the nucleosome's unwrapping dynamics may be disturbed by the crowding under pressure (Donovan, EMBO J, 2019). Our study so far suggests the possibility that crowding by macromolecular accumulation limits protein production by regulating transcriptional machinery.

P1236/B241

Understanding Mitotic Chromosome and Centromere Structure by Direct Micromanipulation.

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The mitotic chromosome is a mechanically and structurally complicated structure that forms the unfolded interphase chromosomes into carefully packaged and self-contained bendable, rod-like structures. In this project, we look specifically at the centromere by tracking CENP-A and CENP-B lengths while applying forces along the longitudinal axis of the chromosome. The CENP-A-based centromere supports the mechanical force of chromosome separation in mitosis and is surrounded by the CENP-B-

rich pericentromere. The constitutively centromeric associated network (CCAN) and kinetochore are built on top of the CENP-A centromere to facilitate chromosome separation. We degraded CENP-C and CENP-N using auxin-inducible degrons, which we verified compromises the CCAN via observation of CENP-T loss. Chromosome stretching revealed that the CENP-A signal does not visibly stretch, including after CENP-C and/or CENP-N degradation. Pericentromeric chromatin deforms by about 3-fold less than the whole chromosome, which also does not change with the loss of CENP-C and/or CENP-N. Chromosome-disconnecting nuclease treatments showed no structural effects on CENP-A. Our experiments show that the core-centromeric chromatin is more resilient than and likely mechanically disconnected from the underlying pericentromeric chromatin, which itself is stiffer than the chromosome arms. We also present our future directions studying SMC complexes, topological proteins, structural densities, and their effects on the whole chromosome mechanics and their interactions with the centromere.

P1237/B242

Survivin Modulates Stiffness-Mediated Proliferation of Glioblastoma Cells.

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Glioblastoma Multiforme (GBM) is an aggressive and lethal form of brain cancer characterized by rapid growth. Pathological stiffening of the extracellular matrix (ECM) triggers hyperproliferation of glioblastoma cells, although the underlying mechanism remains unclear. Survivin, a protein known as an inhibitor of apoptosis and highly expressed in many human cancers, including GBM, plays a key role in regulating cancer cell proliferation and migration. Additionally, survivin is involved in modulating stiffness-mediated cell cycling and proliferation in normal cells, such as vascular smooth muscle cells and embryonic fibroblasts. To investigate whether survivin and ECM genes are highly expressed in human GBM, RNA-sequencing analysis was performed using a publicly available dataset to identify differentially expressed genes (DEGs) present in human GBM tumors as compared to non-tumor samples. Our analysis showed significant upregulation of BIRC5 (the gene encoding survivin), ECM (collagen, lysyl oxidase, and MMPs), and cell cycle-associated (cyclins) mRNAs in human GBM. Gene ontology-based functional enrichment analyses of the upregulated DEGs revealed significant enrichment in cellular components, molecular functions, and biological processes, primarily related to cell cycle progression and the ECM. Thus, we hypothesized that survivin plays a crucial role in regulating stiffness-induced glioblastoma cell proliferation. To assess the impact of ECM stiffness on survivin expression and its role in glioblastoma cell proliferation, we used U87 human glioblastoma cells and cultured them on soft and stiff polyacrylamide hydrogels. Our western blot analysis showed that both survivin and cyclin D1 expression were significantly increased in U87 cells on stiff hydrogels compared to those on soft hydrogels. To further investigate the role of survivin in stiffness-mediated cell proliferation, we inhibited survivin expression in U87 cells cultured on stiff hydrogel with YM155 (a survivin inhibitor) and performed cell counting. At 48 hours, cells treated with DMSO (vehicle control) had double the number of cells compared to minimal change in the YM155-treated condition, indicating that survivin is crucial for stiffness-stimulated U87 cell proliferation. YM155 treatment also reduced cyclin D1 induction in U87 cells cultured on stiff hydrogels. Collectively, our results highlight the crucial role that survivin in stiffness-mediated proliferation of glioblastoma cells. The significant upregulation of survivin and its involvement in cell proliferation on stiff matrices suggest that targeting survivin and its signaling pathways could be promising approach for developing mechanosensitive therapeutic treatments for GBM patients.

P1238/B243

Geometric morphometrics techniques on fibroblast-seeded collagen lattice 3D reconstructions from optical coherence tomography.

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Collagen is a connective tissue that serves many roles in the body, such as providing an environment through which cells can achieve various functions. A cell type closely associated with collagen is the fibroblast, which plays a role in wound healing. Optical Coherence Tomography (OCT), widely used in optometry and other engineering fields, allows for the visualization of semi-lucent structures such as collagen lattices. Geometric morphometrics, which involves the statistical analysis of shapes and their variations, is a powerful technique for analyzing and comparing shapes associated with particular landmarks. The objective of this research was to use OCT to acquire 3D scans of fibroblast-seeded collagen lattices, create 3D reconstructions, and apply geometric morphometric techniques on these reconstructions to determine structural variations in the presence of various treatment groups. A ThorLabs SD-OCT Callisto series was used to acquire 3D scans of the collagen lattices. Key structures analyzed included the top, bottom, and edges of the collagen lattice. Procrustes analysis for landmark alignment and scaling, along with Principal Component Analysis, were used to identify major axes of shape variation. Statistical tests were performed to assess the significance of shape differences. The results indicated that the use of OCT and geometric morphometrics provided a quantitative method for assessing variations in the structure of fibroblast-seeded collagen lattices. These findings enhanced our understanding of the structural properties of the lattices and the effects of various treatments, potentially informing future biomedical applications and treatment strategies.

Protein and RNA Structures

P1239/B244

Cryo-ET Service at CU-Boulder.

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The University of Colorado Boulder Center for cryo-electron tomography (CCET) is a NIGMS NIH-funded service facility for cellular and molecular 3-D imaging and analyses. We are one part of a larger consortium which features a central hub (Univ. of Wisconsin Madison (MCCET), and three spokes. We provide service and training for individual scientists and labs nation-wide. We operate an Aquilos-2 cryo-FIBSEM (Focused ion-Beam Scanning Electron Microscope), a Leica confocal CLEM (correlative light and electron microscopy), plus a long range of relevant peripheral equipment for specimen vitrification and 3-D analysis by cryo-EM. The FIBSEM microscope is our key instrument for vitrified lamellae preparation which will be further imaged in a transmission cryo-EM. The 3-D structure of specimens will, for most parts, be analyzed by tomographic reconstruction. Furthermore, we host numerous trainees for on-site, hands-on training on the FIBSEM, cryo-CLEM, and other relevant technologies.

P1240/B245

Formulating New Cryoprotects Inspired by the Plant Stress Response.

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Dehydrins are proteins expressed in plants during water scarcity, and they play a role in the protection of membranes, DNA, and proteins released due to stress caused by dehydration. Due to their wide variety of binding partners and implications in the stress response of the cell, we hypothesized that the dehydrin from the desert shrub *Ammopiptanthus nanus* (AnAFP) could preserve enzymatic activity during freeze/thaw damage and to promote cell survival during freeze/thaw stress. Current strategies in the cryopreservation of mammalian cell lines rely on the use of chemical components such as dimethyl sulfoxide (DMSO), which can be toxic when used in the clinical setting. In this study we investigated if AnAFP can function as a cryoprotectant. To determine if AnAFP can preserve enzymatic activity during freeze/thaw stress, we flash froze it with the cold sensitive enzyme, lactate dehydrogenase (LDH). Here we show that flash freezing and subsequent thawing with AnAFP preserved LDH enzymatic activity. To determine the effect of AnAFP on mammalian cells during cryopreservation, we report here the results of cryopreservation with AnAFP of various cell lines grown with 2D and 3D strategies. Overall, dehydrins found in the plant stress response can be promising alternatives to traditional cryoprotectants.

P1241/B246

In-situ Cryo-EM Analysis of the Jumbo Phage Infection Cycle in Bacteria.

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The growing number of antibiotic-resistant bacteria is a looming threat for public health. An approach for alternative treatment of bacterial infections is the use of jumbo phages. These lethal viral invaders of bacteria are characterized by a large 200 kb genome that gives room for genetic modifications. This makes them a suitable tool for designing individualized phage therapy that will obviate the use of antibiotics.

Jumbo phage infected bacterial cells harbor a “phage nucleus”, a compartment defined by a proteinaceous shell that encloses the replicating viral genome to protect it from restriction enzymes of the host cell. New studies indicate that this compartment derives from an early phage injection (EPI) vesicle. Upon injection, this vesicle seals the phage DNA and acts as refinery for the synthesis of early phage proteins that fuel the infection cycle and the creation of the nucleus. Even though it’s the most vulnerable stage of infection, so far little is known about the origin of the EPI vesicle and how the phage nucleus evolves.

In-situ electron cryo-microscopy (cryo-EM) is an important tool to investigate jumbo phage infection as it provides insights up to an atomic resolution level. The technique involves rapid freezing of infected bacterial cells and their carving to a thin layer. This layer can then be examined by electron cryo-tomography to visualize macromolecules and cellular compartments.

In-situ cryo-EM revealed that the EPI vesicle, contrary to previous predictions, does not seem to originate from the inner membrane as an evagination upon injection of the phage DNA. Surprisingly, the EPI membrane seems to emanate from the virion and injected into the cytoplasm followed by the DNA. In addition, tomograms reveal the presence of polyribosomes around the EPI, indicating the high metabolic activity of the vesicle as they probably translate early phage proteins. Finally, we have

captured the development of the proteinaceous phage nucleus that involves EPI vesicle deflation as all phage DNA is transferred into the newly formed compartment.
In conclusion, in-situ cryo-EM provides high resolution insights into the jumbo phage infection cycle. Unravelling the jumbo phage visual proteome will enable optimized genetic engineering and usher in the future of customized phage therapy.

P1242/B247

Using Cryo-Electron Tomography to Understand the Dynamics of the HIV-1 Envelope Glycoprotein.

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HIV-1/AIDS is an ongoing epidemic without a cure. Many antiretroviral therapies (ART) mitigate the progression of the disease, but due to the high mutation rate of HIV-1 and the persistence of the infection, drug resistance is a common occurrence over the lifetime of a person with HIV-1. The trimeric glycoprotein found on the surface of HIV-1 virions, Env, is a primary target for antibody therapeutics and vaccine development. However, it has proven to be a challenging target because of the dense glycan shield that surrounds and protects Env. To circumvent this, it would be advantageous to target sites within Env that become accessible during the fusion process. Prior to membrane fusion, it is proposed that the viral and host cell membranes are linked by an extended pre-hairpin intermediate in which the fusion peptide is inserted into the host cell membrane and the transmembrane region is anchored to the viral membrane. The pre-hairpin intermediate state, as well as the process of fusion itself, is challenging to obtain high-resolution structures of in situ due to its transient nature. To overcome this, we will use the highly potent and specific D-peptide entry inhibitor to “lock” Env in the pre-hairpin intermediate state. With Env locked in this fusion state, we will be able to collect thousands of images in the cellular context using cryo-electron tomography. We have optimized a micropatterning technique that can be used to control the morphology of mammalian cells on cryo-EM grids, which will facilitate easier data collection. Additionally, we have implemented protocols in the lab for successful pseudovirus purification and high-throughput characterization.

P1243/B248

Cryo-EM Uncovers Evolutionary Divergence of Filament Assembly in Yeast Asparagine Synthetase Isoforms.

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In budding yeast, the asparagine synthetases Asn1 and Asn2 assemble into micron-scale filaments during nutrient starvation. Asn1 and Asn2 filaments colocalize in stationary phase cells, but Asn1 is required for Asn2 assembly, suggesting an ability for the two isoforms to co-assemble into the same structure. Previous work has demonstrated that cytoplasmic acidification during nutrient starvation may be a common trigger for assembly of filaments by various metabolic enzymes, especially those essential to amino acid and nucleotide homeostasis. Using cryo-electron microscopy (cryo-EM), we identified a single histidine residue critical for Asn1 filament assembly, where its protonation at lower pH drives the formation of a salt bridge with a glutamate on an adjacent monomer. Unexpectedly, by negative stain

EM, we found that Asn1 was not required for purified Asn2 to assemble filaments *in vitro* in a pH-dependent manner. We then solved cryo-EM structures of the Asn2 filament in apo and substrate-bound states at low pH. While the conformation of the Asn2 filament is nearly identical to the Asn1 filament, Asn2 filaments assemble with completely divergent contacts, including a different histidine that interacts with a tryptophan. Cryo-EM structures solved at neutral pH also revealed that Asn1 and Asn2 assemble into identical dimers that use a distinct, non-pH-sensitive assembly interface not found in filaments. Our data suggest that as the pH drops during nutrient starvation, assembly of Asn monomers into filaments is favored over dimer assembly, which may maintain Asn protein levels for starvation recovery. Asn1 and Asn2 also exemplify an intriguing principle of evolution where duplicated genes maintain the same oligomerization interface even as the residues involved change.

P1244/B249

High-order Organization of SUN5 Revealed by Cryo Electron Tomography In Vivo.

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Introduction: SUNs (Sad1p, UNC-84) are fundamental membrane proteins which span inner nuclear membranes as trimers to bind KASH (Klarsicht, ANC-1, Syne Homology) monomers at the outer nuclear membrane. KASH proteins pass the outer nuclear membrane to connect with multiple cytoskeleton filaments. The strong covalent coupling SUN/KASH is crucial to transmit mechanical information from the plasma membrane/cytoplasm to the nuclear envelope and chromatin domains (MCGillivary et al, Current Opinion Cell Biology 2023). SUN5 is the key protein expressed during late sperm differentiation stages which allow the connection of the sperm head to the sperm tail through the proximal centriole, by means of Nesprin3 KASH protein (Manfrevola et al, Genes 2021). Several mutations of SUN5 have been found to cause infertility in men (Xiang et al Reproductive Sciences 2022) shedding light on its importance for a successful animal fertilization. Results: We used recent technological advances in hardware and software for in situ cryo electron microscopy to reveal the structure of SUN5 (~120KDa) from intact human sperm cells forming an unexpected 2D hexagonal lattice at the outer nuclear membrane. We solved the architecture by cryo electron tomography and subtomogram averaging at subnanometer resolution and use the integration of molecular dynamics (MD) simulations and alpha fold modeling to reveal the location of the key amino-acids involved in infertility defects. They are placed at the SUN5 insertion within the inner nuclear membrane and at the intra-trimer and inter-trimer interfaces, being hence crucial for the formation of the lattice. Moreover the distance inner-outer nuclear membrane is strikingly less than 15nm at SUN5s, half the distance than in a canonical human nuclear envelope, due to the mechanical strain induced by the lattice. In addition atomistic MD simulations revealed how the stability of the robust lattice is perturbed introducing patients infertility mutations. Finally we carried out immuno-histochemistry on human tissues to follow SUN5 lattice formation during sperm developmental stages using STORM imaging showing that SUN5 expression and clustering appears only in the late meiotic step. Conclusions: An in situ structure of a small membrane protein at sub nanometer resolution is long awaited and reveals a high-order assembly of a SUN protein (Yerima et al, Biophysical Journal 2023; Jahed et al, Biophysical Journal 2023) and how this organization is fundamental for animal reproduction (Zhang et al, Frontiers in Cell and Developmental Biology 2021). With this work we highlight the importance of in situ integrative structural biology to give novel insights into membrane biology and linked diseases.

P1245/B250

Toward *in situ* Cryo-ET Visualization of RNA and Ribosome Transport on Lysosomes in iPSC-Derived Neurons.

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Polarized cells, such as neurons, require local protein synthesis at metabolically active sites distant from the nucleus. Membrane-less RNA and ribosomes are transported on motile lysosomes via the 'hitchhiking' mechanism, where cargos are docked onto membranous organelles to be indirectly transported along the microtubule networks. Active transport of both lysosomes and hitchhiking cargos is vital to neuronal homeostasis, and dysfunctions of transport adaptor proteins can lead to various neuronal diseases. Live-cell imaging and proteomic studies reveal annexin A11 (ANXA11) as a molecular tether for RNA hitchhiking on lysosomes. However, the mechanism of RNA and ribosome hitchhiking at the molecular level remains largely unknown, mainly due to the lack of high-resolution, direct visualization evidence that characterizes such processes. Furthermore, conflicting conclusions regarding whether ribosomes hitchhike on lysosomes as monosomes or polysomes in axons exist. To observe ribosome and RNA hitchhiking in native axonal environments, we applied cryo-CLEM (Correlative Light and Electron Microscope) and live-cell imaging to target lysosome-mRNA/ribosome co-transport events in an integrated, inducible, and isogenic iPSC-derived neuron cell line. We integrated it with cryo-electron tomography (cryo-ET) to reveal the molecular mechanisms of hitchhiking at unprecedented resolution without chemical fixation. With our established data collection pipeline, template matching followed by subtomogram analysis will be conducted to study the distribution of ribosomes close to motile lysosomes, as well as the tethering mechanisms between lysosomes and the hitchhiking cargos. Integrating cellular structural biological data at different scales in this model system opens up new routes to study fundamental cellular processes and their relationship with the cause of neuronal degeneration diseases.

P1246/B251

Structural Analysis of Lysosomal Proteins via Cryo-Electron Tomography of Affinity-Captured Organelles.

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Lysosomes are essential organelles that maintain cellular energy homeostasis by degrading and recycling cellular components. In addition, lysosomes are now recognized as signaling hubs within the cell. These functions depend on their unique protein composition, which is dynamically remodeled depending on cell state. To fully understand how these proteins operate together, structural information from intact lysosomes is needed, a gap our research aims to fill. Cryo-electron tomography (cryoET) is the only method that can provide this structural information. Obtaining high resolution structures from cryoET remains challenging as it requires averaging of thousands of protein copies. First, one needs to have samples that allow large scale data collections as many of the proteins of interest come in low copy

numbers. Second, raw tomography tilt series traditionally required long and slow processing workflows, often including manual intervention, to obtain high quality and high contrast 3D tomographic volumes. Third, tomograms of whole cells are very crowded with different protein species, making identification of specific proteins of interest difficult. To address these challenges, we developed an affinity-grid system to rapidly capture lysosomes from human cell lysates directly onto electron microscopy grids. These lysosomes retain their acidic luminal pH, verified by lysotracker labeling, a hallmark of functional lysosomes. Combined with our in-house processing pipeline, we can collect and process 2000 high-quality, denoised tomograms in under a week. We have accumulated a dataset of more than 10,000 tomograms, perhaps the largest cryoET dataset of a biological specimen. To identify proteins of interest, we developed two probes for cryo-correlative light and electron microscopy (CLEM). One targets the lysosomal V-ATPase, a key lysosomal protein, while the other is a general label for proteins on organelle surfaces. We are now working on software to automate detection of V-ATPase and other labeled molecules for averaging. These tools will allow structural studies of proteins within any organelle and provide information on functional protein interactions. Additionally, organelle morphology features like size and shape can be measured with high statistical significance given the very large number of tomograms. We will make our data available through the CZ Imaging Institute CryoET Data Portal (https://cryoetdataportal.czscience.com/), which provides biologists and developers open access to high-quality, standardized, annotated tomography data. We hope that the data will serve as a global resource for researchers to gain insights into organelle function.

P1247/B252

Characterization of LRRK2 interactions with cytoskeleton and cellular membranes using Cryo-Electron Tomography.

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Leucine Rich Repeat Kinase 2 (LRRK2) is a large multidomain protein that contains both GTPase and kinase domains. Mutations to this gene are a major cause of genetic Parkinson's Disease. LRRK2 plays critical roles in regulating vesicular trafficking and cytoskeletal dynamics in cells. LRRK2 interacts with the subset of Rab GTPases and gets recruited to trans-Golgi network and endolysosomal membranes. LRRK2 is also known to form extended filaments around microtubules under certain conditions. Although much progress has been made towards structural characterization of LRRK2 in vitro, not a lot is known about how this protein associates with cellular membranes in cells. In this study, we present advances made towards characterizing the architecture of LRRK2 on membranes in their native cellular environment, using correlative light and cryo-Electron Tomography. The insight gained from this study will shed light on LRRK2's mechanism in cells and inform efforts towards therapeutic drug design.

P1248/B253

Mechanism of actin filament severing and elongation by formins.

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Humans express fifteen formins, playing crucial roles in actin-based processes, such as cytokinesis, cell motility, and mechanotransduction. However, the lack of structures bound to the actin filament (F-actin) has been a major impediment to understanding formin function. While formins are known for their ability to nucleate and elongate F-actin, some formins can additionally depolymerize, sever, or bundle F-actin. Two mammalian formins, inverted formin-2 (INF2) and diaphanous-1 (Dia1), exemplify this diversity. INF2 displays potent severing activity but elongates weakly, whereas Dia1 has potent elongation activity but does not sever. Using cryo-electron microscopy (cryo-EM), we reveal five structural states of INF2 and two of Dia1 bound to the middle and barbed end of F-actin. INF2 and Dia1 bind differently to these sites, consistent with their distinct activities. The FH2 and WH2 domains of INF2 are positioned to sever F-actin, whereas Dia1 appears unsuited for severing. Structures also show how profilin-actin is delivered to the fast-growing barbed end, and how this is followed by a transition of the incoming monomer into the F-actin conformation and the release of profilin. Combined, the seven structures presented here provide step-by-step visualization of the mechanisms of F-actin severing and elongation by formins.

P1249/B254

Mechanism of actin filament severing and capping by gelsolin.

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Gelsolin is the prototypical member of a family of Ca^{2+}-activated F-actin severing and capping proteins. A structure of Ca^{2+}-bound full-length gelsolin at the barbed end shows domains G1G6 and the inter-domain linkers wrapping around F-actin. Another structure shows domains G1G3, a fragment produced during apoptosis, on both sides of F-actin. Conformational changes that trigger severing occur on one side of F-actin with G1G6 and on both sides with G1G3. Gelsolin remains bound after severing, blocking subunit exchange.

P1250/B255

MARTINI WITHOUT THE TWIST: UNVEILING A MECHANICALLY CORRECT MICROTUBULE THROUGH BOTTOM-UP COARSE-GRAINING IN MARTINI 3.

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Microtubules are essential cytoskeletal filaments involved in cell motility, division, and intracellular transport. These biomolecular assemblies can exhibit complex structural behaviors influenced by various biophysical factors. However, simulating microtubule systems at the atomistic scale is challenging due to their large spatial scales. Here, we present an approach utilizing the Martini 3 Coarse-Grained (CG) model coupled with an appropriate elastic network to simulate microtubule-based systems accurately. By iteratively optimizing the elastic network parameters, we matched the structural fluctuations of CG hetero-dimer building blocks to their atomistic counterparts, thereby reproducing close-to-experiment mechanical properties and structural details. Our efforts culminated in a ~ 200nm microtubule built with

~ 6 million interaction-centers. This CG simulation framework enables the exploration of macroscopic biophysical phenomena at a microscopic level, offering valuable insights into the mechanisms underlying microtubule-associated processes in cellular biology. With MARTINI 3 CG simulations, we can bridge the gap between computational efficiency and molecular detail, enabling investigations into these biophysical processes over longer spatio-temporal scales with amino acid-level insights.

P1251/B256

Molecular insights for activation and filamentation of human glutaminase.

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Glutaminase serves as a key rate-limiting enzyme in glutaminolysis, converting glutamine into glutamate, which subsequently enters the tricarboxylic acid (TCA) cycle. Beyond its metabolic role, glutaminase is also crucial in maintaining redox homeostasis, regulating autophagy, supporting the immune system, maintaining central nervous system function, and facilitating senolysis. The allosteric regulation of glutaminase, therefore, is a compelling topic with far-reaching implications for understanding glutamine metabolism and associated diseases.
Phosphate was identified as a natural agonist of glutaminase in 1947, and filamentation of glutaminase, first observed in 1960, has been recognized for its influence on enzyme activity and metabolic homeostasis. While both regulatory mechanisms are thought to play crucial roles in glutaminase function, their precise modes of action and underlying mechanisms remain unclear. In this study, we used cryo-electron microscopy to determine the filament structure of human glutaminase in complex with phosphate. Our high-resolution structure reveals that phosphate binds at the dimer-dimer interface, uncovering an allosteric activation mechanism facilitated by filamentation and remodeling of the catalytic pocket.
Remarkably, we found that phosphate counteracts the inhibitory effects of BPTES (a classical antagonist) and CB-839 (a compound currently in phase II clinical trials) on enzyme activity and filamentation. The precise mapping of phosphate binding sites and insights into the regulation of filamentation lay a crucial foundation for the rational design of glutaminase agonists and antagonists with significant therapeutic potential.

P1252/B257

Klf4 Chooses DNA to Bind: Local Energy Landscape.

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The billion-basepair DNA polymer in the nucleus produces astronomical complexity when diverse cell types emerge from transcription factors (TFs) binding to small regions of DNA. Advanced assays have given us significant insights into protein-DNA binding in vitro and in vivo, yet the mechanisms underlying the robust specificity of transcription factors for particular short DNA sequences remain unclear, given the complexity of TF-DNA and TF-TF interactions. Here, we performed high-throughput in vitro binding

energy measurements of TF and short DNA oligonucleotides (oligos) using a fluorescence anisotropy competition assay. We used one Yamanaka factor, Krüppel-like factor 4 (Klf4), a TF that is known to self-interact. A designed set of oligos, each 17 base pairs long, was chosen. The binding energy of Klf4 to oligos from the set was measured relative to two distinct competitor oligos, weak and strong binders to Klf4. The competitor oligo was fluorescently labeled at a fixed concentration, while the other was unlabeled, with concentrations increasing over a 1000-fold range up to 50 μM. After the physical removal of single-stranded DNA, the simplest model, Langmuir adsorption, with one binding relative energy fitting parameter for each oligo, captured the data well. One can infer a binding energy relative to a weak competitor, knowing the energy relative to the strong one, indicating the consistency of the method. Measurements revealed that the data could not be fully captured by the simplest linear additive base pair model. Instead, different energy matrices emerged when applying this model to the energy vicinity of the labeled oligos. We conclude that the energy landscape is described linearly only in local regions of sequence space. Using the energy landscape to compare Klf4 binding to long DNA helps us learn the difference in binding to oligos and long DNA, manifesting a next step towards understanding this in vivo. The model characterizing the local TF-oligo energy landscape is a step towards understanding the complexity of TF-DNA interactions in the nucleus.

P1253/B258

Biochemical characterization of BLOC-2 in melanosome maturation.

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Lysosome-Related Organelles (LROs) are a diverse group of cell-type specific, membrane-bound subcellular organelles. Members of this family share some features with lysosomes, but they are functionally and morphologically distinct and contain tissue-specific constituents that confer specific functions that are important for the organism. One example is the melanosome, LRO in which melanin pigments are synthesized and stored in eye pigment cells and in melanocytes of the basal layer of the epidermis and the hair bulb of hair follicles. The melanosome is one of the most common models to study and understand the molecular mechanisms underlying the formation and maturation of LROs. The impairment or absence of melanin underlies oculocutaneous albinism, a feature of the Hermansky-Pudlak syndromes (HPS). HPS is a group of generally rare genetic disorders of intracellular vesicle trafficking that disrupt the function of several LROs including melanosomes. Mutations in any of at least 11 genes underlie the disease; these genes encode subunits of four protein complexes, AP-3 and BLOC-1, -2, and -3. Three of the characterized HPS subtypes result from mutations in subunits of BLOC-2, a protein complex for which the molecular function remains unknown. To better understand the role of BLOC-2 in melanosome maturation we aim to elucidate its three-dimensional structure using cryo-EM. We utilized the baculovirus expression system to express the three subunits of BLOC-2; HPS3, HPS5 and HPS6, in Sf9 cells, and affinity purified the complex using FLAG-tag on the HPS5 subunit. The complex was further purified by glycerol gradient sedimentation with cross-linking, demonstrating that the three subunits were forming the complex. Mass photometry analysis suggests that the purified BLOC-2 is a well-defined particle with a size consistent with either a monomer or dimer of the heterotrimeric complex. Our ongoing efforts are focused on elucidating the three-dimensional structure by cryo-EM and identifying its interacting partners.

P1254/B259

Biochemical Basis of SMYD5 in HIV Latency and Transcriptional Reactivation.

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SMYD5, a protein lysine methyltransferase, has been shown to regulate HIV-1 reactivation from latency. However, the biochemical mechanisms underlying this regulation remain unclear. This study aims to uncover these mechanisms by focusing on SMYD5's interactions with Transactivation Response RNA (TAR RNA) and the viral Tat protein. We hypothesize that SMYD5 influences the assembly of the transactivation complex at the HIV promoter, potentially by inducing conformational changes in TAR RNA. Gel shift assays using both full-length and truncated SMYD5 mutants revealed that SMYD5 binds weakly to TAR RNA. Consistent with this, AlphaFold3 predictions of the SMYD5-Tat-TAR RNA complex structure showed that the interaction between SMYD5 and TAR RNA is modest. In contrast, Tat binds extensively to SMYD5, with lysine 71 positioned near the SAM molecule, suggesting that SMYD5 could methylate this lysine residue. GLO methyltransferase assays confirmed that SMYD5 does not methylate TAR RNA, supporting the idea that SMYD5 primarily acts as a binding partner. In conclusion, these findings highlight the critical role of SMYD5 in modulating HIV-1 transcription via its interaction with TAR RNA and Tat, making it a promising target for strategies to reactivate latent HIV.

P1255/B260

Single-molecule Spectroscopy of Apolipoprotein E Reveals a Complex Conformational Ensemble.

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The e4-allele variant of Apolipoprotein E (ApoE4) is the strongest genetic risk factor for Alzheimer's disease, though it only differs from its neutral counterpart ApoE3 by a single amino acid substitution. While ApoE4 influences the formation of plaques and neurofibrillary tangles, the structural determinants of its pathogenicity remain undetermined due to limited structural information. Previous studies have led to conflicting models of the C-terminal region positioning with respect to the N-terminal domain across isoforms, largely due to the potential contribution of data representing heterogenous oligomers in solution. Here, we apply a combination of single-molecule spectroscopy and molecular dynamics simulations to construct an atomically-detailed model of monomeric ApoE4 and probe the effect of lipid association. Importantly, our approach overcomes previous limitations by allowing us to work at picomolar concentrations where only the monomer is present. Our data reveal that ApoE4 is far more disordered and extended than previously thought and retains significant conformational heterogeneity after binding lipids. We further investigate the impact of key mutations by inserting serine residues that mimic the ApoE3 and ApoE2 isoform sequence differences, C112S and C112S C158S, respectively. Comparing the proximity of the N- and C-terminal domains across the three major isoforms (ApoE4, ApoE3, and ApoE2) suggests that all maintain heterogenous conformations in their monomeric form, with ApoE2 adopting a slightly more compact ensemble. Inspection of local conformational changes across different domains, in the context of the full-length protein, reveals that the major difference across isoforms occurs in the conformations adopted by the C-terminal tail. Overall, these data provide a new foundation for understanding how ApoE differs from non-pathogenic and protective variants of the protein.

P1256/B261

The impact of crowding on the conformations and interactions of the disordered proteins and RNA.

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The cellular milieu is crowded with a significant concentration of macromolecules and metabolites. Such a crowded environment affects protein and nucleic acid properties. In particular, disordered proteins and unstructured RNA are very sensitive to a crowded environment due their flexibility. Here, we investigate the effect of crowding on the disordered N-terminal domain (NTD) and RNA Binding Domain (RBD) of the SARS-CoV-2 Nucleocapsid protein as well as archetypal RNAs as a case study to quantify crowding's impact on protein and nucleic acid conformations, as well as interactions between the two, using single-molecule fluorescence spectroscopy.Previously, we have shown that the NTD is flexible and dynamic and facilitates RBD-recruitment of RNA. We mimic the cell's crowded environment by titrating increasing concentrations of polyethylene glycol (PEG). Single-molecule Förster Resonance Energy Transfer provides a direct measure of the associated conformational changes. We found that large molecular weight PEG induces a collapse of the disordered NTD, whereas small molecular weight PEG leads to an expansion. Under both conditions, nanosecond Fluorescence Correlation Spectroscopy confirms that the chain remains dynamic. Data can be explained by accounting for two opposing effects: i) favorable interactions between the protein and crowders that locally increase the effective excluded volume of the protein and ii) screening of excluded volume interactions by crowders. Only a collapse was observed for rU_{40} for all PEGs tested.We also characterized the protein-RNA interaction in the presence of crowding agents. While for all PEG molecules tested, we observed an increase in the binding affinity of the protein, the trend is not monotonic as a function of the degree of polymerization, suggesting additional impact of non-specific protein-PEG interactions on binding. To separate the enthalpic and entropic contributions introduced by the crowders, we investigated the temperature dependence of binding in absence and presence of crowders.Overall, our data provide new insights into understanding and modeling the contribution of crowding effects on disordered proteins, including the impact of interactions between proteins and crowders.

P1257/B262

Physical-chemical probing reveals a nanoscale heterogeneous condensate structure in live cells.

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Biomolecules cluster together to create unique subcellular microenvironments called condensates, which have roles that include nucleic acid production, maintenance, and assembly with proteins. The physical principles underlying condensate form and function are controversial, in part because we lack methods to determine condensate structure in cells. Here, we employ the principles of physical chemistry to develop an approach we term physical-chemical probing, enabling the determination of the local nanoscale structure within condensates in live cells using quantitative microscopy. Two specific applications are developed employing basic principles of polymer confinement and scaling laws. The first, called Local Size Exclusion, can measure the local mesh size around a protein within a condensate. In the second, denoted Molecular Lasso, we can determine the distance preferences between specific proteins within a condensate. We apply these approaches to the nucleolus, the largest condensate in cells, as a proof of principle and find that the nucleolus is not uniform but contains a wide heterogeneity of interactions and meshwork sizes that correlate with ribosome biogenesis. Our results suggest that